GASTROINTESTINAL BACTERIAL SYMBIONTS: REPRODUCTIVE STRATEGY AND COMMUNITY STRUCTURE

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The aim of this thesis is to describe symbiotic gastrointestinal bacterial populations in relation to their environment, both at the community and cellular level, in order to advance the field of microbial ecology. Two different levels of relations are examined: in bacterial populations as they undergo differentiation, and in bacterial symbiotic communities in relations with a host. Bacterial populations routinely collaborate in order to fully differentiate in response to environmental stress. The literature review herein describes the current state of knowledge about bacterial programmed cell death during differentiation. This study found that programmed lysis of the Low G+C Gram positive bacterial symbiont *Epulopisciumo*ccurs after marked DNA replication in the terminally differentiated mother cell, as well as the expected replication in developing offspring cells. This energetically expensive process sustains the large metabolically active cell and may allow nutrient storage in the form of DNA for later use by the offspring or host. The close relative of *Epulopiscium, Metabacterium polyspora*, has different DNA dynamicsand reproduces through the formation of multiple dormant endospores. In model systems DNA replication is blocked after induction of sporulation, In contrast, *M. polyspora*replicates DNA inside developing forespores. This may allow *M. polyspora*to modulate the number of forespores produced based on local nutrient availability within the gastrointestinal tract of its host. Both *Epulopiscium* and *M. polyspora*appear to have evolved

reproductive strategies and related DNA replication dynamics that are suited to their particular relationship with the host. Studies of the gastrointestinal microbiota of the coral reef fish *Pomacanthus sexstriatus* also illustrate the ability of organisms to shape and be shaped by their environment. The unusual prevalence of sulfate reducing bacteria in this community could aid host digestion of a wider range of algae, including those that use sulfonated carbohydrates as a defense against predation. This ability may have an impact on larger coral reef ecology, promoting coral reef resilience by controlling fast-growing algal populations. In summary, investigations into these bacterial populations and communities provide data that can only be explained when bacteria are placed within the context of their environment and evolutionary history.

BIOGRAPHICAL SKETCH

Rebekah Ward attended Georgia State University from 1997-2003, receiving her Bachelor of Science degree from the Department of Biology. She went on to graduate studies at Cornell University in the Department of Microbiology, joining the lab of Dr. Esther Angert in 2004. She will be starting a Postdoctoral fellowship in the lab of Dr. Charles Moran of Emory University after completion of her PhD.

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CHAPTER 1

BACTERIAL PROGRAMMED CELL DEATH DURING DIFFERENTIATION: A LITERATURE REVIEW

Summary

Complex multicellular life relies on an ability to regulate the destruction of unwanted cells with a process of programmed cell death (PCD) during development and homeostasis. Data from several groups of bacteria indicate that an analogous process can occur at certain stages of bacterial differentiation. The developmental program of endospore formation found in *Bacillus* and Clostridia exhibits some of the hallmarks of bacterial PCD during regulated lysis of the mother cell. *Myxococcus* fruiting body formation requires lysis of a majority of the cells within a population in order to successfully complete myxospore formation and dispersal. Colonies of the Actinobacterial genus *Streptomyces* exhibit a massive lysis of vegetative cells coordinated with the formation of resistant cells specialized for dispersal. Development in these bacterial populations also has defined and highly regulated DNA dynamics, including checkpoints involving DNA replication and segregation and the production of DNases coordinated with cell lysis.

Introduction

Programmed cell death (PCD) is a regulated cell death process that is required for homeostasis and development in multicellular organisms (Jacobsen and McCathy, 2002). It differs greatly from necrosis, or unregulated cellular disintegration, both in morpholohical characteristsics and in impact on the whole organism. The most well characterized form of PCD is apoptosis during which cells go through a regular series of morphological changes which are associated with the action of certain cell death regulators known as caspases. The first stages of apoptosis are cell shrinkage and

DNA condensation. The nuclear DNA becomes tightly associated with the nuclear membrane. The membrane system becomes higly invaginated, a phenomenon known as "blebbing." If phagocytosis does not occur, small vacuoles filled with chromatin and organelles, apoptotic bodies, may form. Eventually, the apoptotic cell will be ingested by other cells. This system avoids the activation of an immune response to lysed cellular material and allows orderly cellular destruction within a multicelluar environment (Jacobsen and McCathy, 2002).

Until recently, canonical programmed cell death was assumed to have emerged with the evolution of multicellular life (Ameisen, 2002). Unicellular life was considered incapable of evolving a mechanism of PCD because the cell exhibiting the phenotype would be unable to pass the genes involved onto progeny. But examples of bacterial PCD have been well documented for the past 40 years (Ameisen, 2002). Key to reconciling the data with the prevailing concepts of evolution was the recognition of population-level interactions, bacterial sociality (Shapiro, 1998). Individual bacterial cells routinely collaborate during differentiation to form specialized cell types. Often this process involves the transition from vegetative growth to provisional differentiation processes for the purposes of dispersal (Shapiro, 1998). This transition has been characterized as akin to the distinction between eukaryotic somatic and gametic cell types (Ameisen, 2002). In the context of population-level survival strategies, evolution of "cellular suicide" among single celled organisms is no longer counter-intuitive. What follows is a short review of the studies of bacterial programmed cell death during colony differentiation with particular emphasis on DNA dynamics and fate.

Bacillus

The genus *Bacillus* of the bacterial phylum Firmicutes is composed of rod-shaped aerobes, or facultative anaerobes, many of which are capable of endospore formation.

These heterotrophic bacteria are found in a wide variety of habitats from soil, to rocks (endolithic bacteria), to gastrointestinal systems of insects (Nicholson, 2002). Although *Bacillus* reproduces through binary fission, sporulation is initiated in response to environmental stress and allows the creation of a dormant resistant endospore (Errington, 2003). The single endospore created by each *Bacillus* cell can then go on to germinate under more favorable environmental conditions and return to vegetative growth (Setlow, 2003).

Differentiation Program

Sporulation is induced by a number of factors, including nutrient deprivation and high cell density, in the model *Bacillus*, *B. subtilis* (Sonenshein, 2000). The activation of the regulatory protein Spo0A initiates a cascade of events involving the asymmetric division of the sporulating cell. In an apparent modification of vegetative cell division, a septum is constructed near one pole of the cell, forming a forespore compartment and a mother-cell compartment (Errington, 2003). This partition allows differential transcriptional programs leading to different cell fates. After transcription by the early sigma factors, σ^F in the forespore and σ^E in the mother cell, the cells are irreversibly committed to sporulation, regardless of changes in environmental conditions (Dworkin and Losick, 2005). Through cell-to-cell communication and the production of compartment-specific proteins, the mother cell and forespore collaborate on building a dormant, resistant endospore. The mother-cell membrane engulfs the forespore and a spore cortex of modified peptidoglycan is built. A proteinaceous spore coat synthesized by the mother cell and the interior of the forespore is partially dehydrated (Stragier and Losick, 1996). This elaborate process of differentiation is followed by mother-cell lysis and mature endospore release.

Programmed Cell Death

Although there are systems which have been described in *B. subtilis* that affect lysis of cells prior to sporulation (Gonzalez-Pastor *et al.*, 2003), programmed cell death of the mother cell upon completion of sporulation will be the focus herein
. A recent cytological investigation into PCD of sporulating *B. subtilis* has begun to characterize the process of mother-cell lysis (Hosoya *et al.*, 2007). After spore maturation, the mother cell undergoes a sequential deterioration, beginning with membrane disruption. Intense globular membrane staining is seen at one or both poles of the cell. This is followed by a loss of DNA staining in the mother-cell compartment. In the final stages of mother-cell degradation, the activity of autolysins dismantles the mother-cell wall, completing the process. The exact molecular mechanisms of membrane disruption, potentially a key step in mother-cell degeneration, remain unknown.

Autolysins have been identified which contribute to mother-cell lysis in *B. subtilis* (Smith *et al.*, 2000). Two of these, CwlC and CwlH, are developmentally regulated, produced during sporulation by the late-sporulation transcription factors σ^K and GerE (Kuroda *et al.*, 1993; Nugroho *et al.*, 1999). Single mutants of either CwlH or CwlC do not significantly inhibit mother-cell lysis, but double mutant mother cells fail to release the mature endospore even after 7 days (Nugroho *et al.*, 1999). Another autolysin, LytC, also known as CwlB, contributes to mother-cell lysis, despite the fact that CwlB is no longer transcribed in stationary phase or sporulating cells (Smith and Foster, 1995). It appears that residual CwlB is active in sporulating cells well after expression of this gene has ceased. Data from zymography analysis and the phenotypes of multiple mutant strains indicate that CwlC is the major autolysin responsible for mother-cell lysis, although full efficiency of lysis requires the activity of either CwlH or CwlB as well (Nugroho *et al.*, 1999). Strains with mutant copies of *cwlB* and *cwlH* were only weakly defective in mother-cell lysis. These autolysins are

not unique to *B. subtilis*. CwlC and CwlH have putative homologs in other *Bacillus* species such as *B. amyloliquefaciens* (76% amino acid identity with CwlC over the length of the protein and 56% with CwlH), and *B. pumilus* (65%, and 62%), (Ward, unpublished data). Cross-reactivity to *B. subtilis* anti-CwlC was also seen for multiple *Bacillus* species, suggesting that some of these sporulation-specific autolysins may be conserved throughout the genus (Smith and Foster, 1995).

There have been contradictory reports of both the timing of CwlC expression as compared to mother-cell lysis and the cellular location of CwlC. The most recent data indicates that this protein is secreted, although no detectable signal sequence has been observed (Smith and Foster, 1995). The authors found that significant expression occurred six hours after (t_6) sporulation began whereas mother-cell lysis did not occur until t_8. This raised the possibility that there is additional regulation of mother-cell lysis. Candidates for direct or indirect regulation include CwbA, SpoIID, and YabG. CwbA has been shown to stimulate the activity of both vegetative and sporulation-specific autolysins, including CwlC (Kuroda and Sekiguchi, 1992). CwbA appears to be co-transcribed with CwlB, and thus is under the control of σ^D, suggesting that it may have only a limited role in activation of these late-stage sporulation autolysins (Kuroda *et al.*, 1993). The alternative sigma factor, σ^D, is expressed late in expontential phase and is involved in chemotaxis, motility, and falgellar synthesis (Kuroda and Sekiguchi, 1992. SpoIID is a σ^E-encoded gene that plays a role in engulfment of the forespore and prevention of the formation of septa at both poles of a sporulating cell (Errington, 2003). SpoIID has homology to CwbA although there is little data on the direct effect of SpoIID on CwlC, CwlB, or CwlH (Kuroda and Sekiguchi, 1992). Recent work revealed that one of the factors contributing to increased vegetative cell lysis in Spo0A mutants upon reaching stationary phase was the decreased production of extracellular proteases. This decrease correlated with

increased stability of the major autolysin CwlB (Kodama *et al.*, 2007). YabG is an extracellular protease that is part of the Spo0A regulon and thus may have some role in controlling premature lysis of mother cells (Molle *et al.*, 2003). As premature mother-cell lysis would have disastrous consequences for successful sporulation, the need for tight regulation of these autolysins is self-evident.

However, the biological consequences of a complete failure to lyse have not been explored. All published data on spore properties done in autolysin mutant backgrounds have involved procedures which physically lyse the mother cell prior to examining germination efficiency and spore resistance (Kuroda *et al.*, 1993; Nugroho *et al.*, 1999; Smith and Foster, 1995). Presumably, a failure to lyse would hinder dispersal of the endospore to more favorable conditions (Lewis, 2000). Intact mother-cell material surrounding the spore might also interfere with germination, not only as a physical constraint, but also by interfering with access to signals for germination (Setlow, 2003). Despite a wealth of data about the molecular mechanisms of most stages of sporulation and germination, questions still remain about mother-cell lysis.

DNA Dynamics

Bacillus subtilis requires tight control over DNA replication, chromosome segregation and partitioning in order to successfully undergo differentiation (Hilbert, 2004; Piggot, 2004). Sporulation cannot proceed in cells where DNA replication is impaired; there is a strict requirement for two chromosomes (Burkholder *et al.*, 2001). After induction of sporulation, additional DNA replication is tightly repressed (Burkholder *et al.*, 2001; Castilla-Llorente *et al.*, 2006). During asymmetric division of the cell the chromosomes form an axial filament stretching the length of the cell with the origins tethered to the poles (Ben-Yahuda, 2003; Ireton, 1994; Quisel, 2000; Wu, 2003). The origin-proximal 30% of the *B. subtilis* chromosome is captured inside the forespore compartment and the remaining 70% of the chromosome is subsequently transferred

from the mother-cell cytoplasm into the forespore (Errington, 2003). This temporal and spatial regulation of the chromosomes is required for the compartmentalized production of alternative sigma factors. These mediate differential gene expression that contributes to the disparate fates of the mother cell and the spore. Late in sporulation, the forespore-associated chromosome undergoes packaging in small acid soluble proteins (SASPs) that protect the DNA from damage and speed germination (Setlow *et al.*, 2000). The mother-cell chromosome is fated for degradation and release into the environment. The σ^K-regulated extracellular DNase NucB facilitates the breakdown of mother-cell DNA (Hosoya *et al.*, 2007). It appears that mother-cell lysis begins with membrane rupture that may provide access to NucB for its substrate, potentially allowing DNA degradation even prior to the breakdown of the mother-cell wall material (Hosoya *et al.*, 2007). The ultimate fate of this fragmented DNA remains obscure, although some authors have suggested that it may serve as an energy source for sporulating kin (Hosoya *et al.*, 2007; Lewis, 2000).

Clostridia

Many members of the order *Clostridiales* of the phylum Firmicutes are also capable of endospore formation. This group is historically defined as non-sulfate reducing, fermentative, and strictly anaerobic bacteria. They are capable of fermenting substrates as diverse as ethanol, purines, amino acids and carbohydrates into end products such as fatty acids and CO_2 (Durre, 2005). Many described Clostridia are roughly rod shaped, reproduce through binary fission, and form a single endospore (Angert, 2005; Paredes, 2005). Within the Clostridia which produce a single endospore, the size, shape, and cellular location of the endospore varies between different species (Durre, 2005). Clostridia are capable of solventogenesis concomitant with sporulation and applications for this property in biotechnology have been extensively explored (Durre, 2005).

Differentiation Program

Compared to their relatives in *Bacillales,* little information is available on the molecular biology of Clostridial development. However, recent advances have been made using bioinformatics (Paredes, 2005) and studies in the model Clostridia, *C. acetobutylicum* (Durre and Hollergschwandner, 2004). It appears that much of the intermediate stages of endospore formation is conserved between *Bacillales* and *Clostridiales* but there are major differences in initiation (Paredes, 2005). The most common environmental stress signaling Clostridial sporulation is not nutrient limitation, as in *B. subtilis,* but instead a decrease in pH and the potential for dissipation of the proton motive force over the cytoplasmic membrane (Durre and Hollergschwandner, 2004). Many Clostridia also undergo differentiation even prior to the induction of sporulation, swelling and forming the "clostridial stage" cells that appear phase bright under the microscope. During this transition they accumulate linked glucopyranose units called granulose that serve as a carbon and energy source during sporulation. However, the transcriptional cascade in Clostridia that allows temporal and spatial regulation of gene expression in the mother cell and forespore compartments remains similar to that of *Bacillales.* The core genetic complement required for the late stages of sporulation in *B. subtilis* is not well conserved among Clostridia (Paredes, 2005).

Also, there are several genera within the order *Clostridiales* that form multiple endospores, and thus have alternative molecular mechanisms for endospore formation (Angert, 2005). *Metabacterium polyspora* creates anywhere from one to nine endospores per mother cell and uses sporulation as its primary means of propagation (Angert *et al.,* 1996; Angert and Losick, 1998). *Anaerobacter polyendosporus,* a highly pleomorphic Clostridia, can produce from one to seven endospores depending on culture conditions (Siunov *et al.,* 1999). *Epulopiscium* and *Epulopiscium*-like

symbionts of surgeonfish are morphologically diverse and produce single or multiple live offspring or single or multiple endospores as well (Angert *et al.*, 1993; Angert and Clements, 2004; Flint *et al.*, 2005). One commonality among all *Clostridiales* studied thus far is that mother-cell lysis is required for endospore or offspring release (Angert, 2005; Durre and Hollergschwandner, 2004).

Programmed Cell Death

There are reports from studies on *C. acetobutylicum* P262 that during industrial fermentation a segment of the population lyses just prior to sporulation (Jones *et al.*, 1982). This system may be reminiscent of the sporulation killing factor programmed cell death mechanism described in *B. subtilis* (Gonzalez-Pastor *et al.*, 2003). However, little is known about the mechanisms of Clostridial mother-cell lysis upon completion of sporulation. NCBI Blast searches using *B. subtilis* CwlC, CwlB, and CwlH come up with only a handful of hits with any significant similarity to these known late-sporulation autolysins (Ward, unpublished data). In at least one Clostridia, *C. difficile,* the CwlB gene has strong similarity to *B. subtilis* but the protein is used for adhesion during pathogenesis (Waligora *et al.*, 2001). The cytology of mother-cell death has been described in *Epulopiscium* sp type B (Ward, under review by DNA and Cell Biology). The process appears to involve the disruption of DNA organization that coincides with accumulation of blebs and irregular staining of the cell membrane. Cell wall material is degraded in the mother cell and large gaps appear in the wall in late stages of development. These morphological changes are reminiscent of the stages seen in canonical programmed cell death (eukaryotic apoptosis).

DNA Dynamics

Transcriptional analysis using microarray data from *C. acetobutylicum* suggests that a two-fold down regulation of *dnaA* occurs upon differentiation into the clostridial form (Alsaker and Papoutsakis, 2005). This repression of *dnaA* continues throughout the

rest of sporulation in this species, suggesting that some Clostridia may share the *B. subtilis* Spo0A mediated repression of DNA replication (Jones *et al.*, 2008). Many bacteria within *Clostridiales* form an axial filament at the onset of sporulation, presumably to ensure proper chromosome capture in the forespore (Durre, 2005). There are several Clostridia with putative homologues (amino acid identity above 40%), to the core *B. subtilis* proteins involved in tethering the origins of the chromosomes to the poles during formation of the axial filament (DivIVA, Soj, Spo0J)(Ward, unpublished data). Transcriptional analysis of *C. acetobutylicum* revealed that some of these putative homologues are developmentally regulated (Jones *et al.*, 2008). SpoIIIE, the *B. subtilis* DNA translocase responsible for transport of the chromosome into the forespore, also appears to have putative homologues with similarity (40-60% identity) among many Clostridia (Ward, unpublished data). Because the origin tether complex helps to determine the polarity of SpoIIIE chromosome transport in *B. subtilis* (into or out of the forespore) it appears that Clostridia may have conserved this elegant mechanism of chromosome partitioning (Becker and Pogliano, 2007). Additionally, there are data that putative homologues of small acid soluble proteins that protect the endospore genome are developmentally regulated in *C. acetobutylicum* (Jones *et al.*, 2008).

However, DNA dynamics become more complex in organisms that form multiple endospores. *Metabacterium polyspora* appears to have evolved a mechanism of modulating the number of endospores produced (anywhere from one to nine) by altering DNA replication dynamics found in *C. acetobutylicum* and *B. subtilis* (Ward and Angert, 2008). By detection of the nucleotide analogue bromodeoxyuridine, incorporated during development, it was revealed that as forespores grow in size, they contain an increasing proportion of newly replicated sporangial DNA. Replication inside the developing forespores could allow *M. polyspora* to maximize its

reproductive fitness by modulating the number of forespores produced based on up-to-date information about local nutrient availability. Data from *Epulopiscium* sp. type B also indicates that offspring are capable of DNA replication prior to release from the mother cell (Ward, under review by DNA and Cell Biology). However, the more remarkable finding in this organism was the massive quantity of newly replicated DNA in terminally differentiated mother cells that are fated for lysis. This DNA replication inside the mother cell may be characterized as somatic, as these chromosomes do not appear to act as informational molecules for the next generation. The additional chromosomes could be serving a metabolic rather than genetic purpose. They may be required to support the metabolic activity of these large and growing bacteria. Alterantively, the DNA may serve as an energy storage molecule with timed release to aid newly emerged offspring. These variations within the order *Clostridiales* suggest that the core machinery of endospore formation has been modified over time to adapt to the needs of specific ecological niches.

Myxococcus

The order *Myxococcales* belongs to the δ-proteobacteria, and is related to sulfate reducing lineages of the *Desulfovibrionacae* as well as to the bacterial parasite *Bdellovibrio* (Dworkin, 1996). The *Myxococcales* are soil-dwelling bacteria that are distinguished by their remarkable sociality. While some Myxobacterial genera are cellulolytic, other types hunt prey bacteria through collaborative swarming and cooperative utilization of digestive enzymes (Kaiser, 2003). The life cycle of the model organism *Myxococcus xanthus* can include both vegetative growth and differentiation into mounds of cells with spores at the top called fruiting bodies (Dworkin, 1996). Three factors influence Myxococcal differentiation: cell density, nutrient limitation, and the presence of a solid surface upon which to build the multicellular structure (Sogaard-Andersen *et al.*, 2003).

Differentiation Program

As in *B. subtilis*, gene expression in *Myxococcus xanthus* is spatially and temporally regulated so that morphogenesis is tightly controlled (Kroos, 2007). One of the special challenges faced by *Myxococcus* is the coordination of appropriate gene expression in tens of thousands of cells, as opposed to the two physically attached differentiating cell types found in *B. subtilis* (Kroos, 2007). These thousands of cells utilize cell-to-cell signaling and relative cellular position to regulate gene expression (Sogaard-Andersen *et al.*, 2003). The developmentally regulated cell surface protein, C-signal, is a key component of the differentiation cascade leading to different cell fates within the *Myxococcus* colony. During aggregation, cells align end-to-end, increasing the exposure to the pole-enriched C-signal. Cells in the center of the aggregate are thus exposed to the high-levels of C-signal required for late-development gene transcription and sporulation. C-signal induces three different responses: activation of a response regulator, FruA (involved in aggregation), regulation, in concert with FruA, of at least 53 genes during development, and upregulating C-signal expression (Gronewold and Kaiser, 2001; Sogaard-Andersen *et al.*, 2003). The modular responses to different levels of C-signal allow the spatial position of individual cells within the colony to determine developmental fate (Kroos, 2007; Sogaard-Andersen *et al.*, 2003). While the cells at the center of the fruiting body form resistant myxospores, well adapted for dispersal, the remaining cells in the fruiting body, about two-thirds of the colony, undergo lysis (Dworkin, 1996; Kroos, 2007).

Programmed Cell Death:

Recent work in *M. xanthus* has described an elegantly regulated system of programmed cell death using the bacterial toxin MazF (Nariya and Inouye, 2008; Sogaard-Andersen and Yang, 2008). MazF has been characterized as a programmed

cell death effector in *E. coli* that responds to signals such as starvation and DNA damage (Engelberg-Kulka *et al.*, 2005; Engelberg-Kulka *et al.*, 2006). MazF and its cognate antitoxin MazE are found in a variety of bacteria and appear to play an important role in bacterial stress responses (Pandey and Gerdes, 2005). *M. xanthus* lacks the antitoxin MazE and instead, MazF is held inactive through association with the unphosphorylated transcription factor MrpC (Nariya and Inouye, 2008). Unphosphorylated MrpC activates *mrpC* and *mazF* transcription. Early in development, a protein kinase cascade phosphorylates cellular MrpC not bound to MazF, thus inactivating free MrpC. Late in development, proteolysis of MrpC occurs forming a product MrpC2 that is insensitive to phosphorylation. MrpC2 induces the transcription of both *mazF* and *mrpC* and the release of MazF from MrpC (Nariya and Inouye, 2008). Massive autolysis of the cells in the stalk of the fruiting body follows. The proper functioning of this system of PCD is required for efficient sporulation; MazF mutants were defective in fruiting-body formation and produced only 8% of the normal complement of spores (Sogaard-Andersen and Yang, 2008). There is evidence that the nutritional status of the colony may affect the extent of lysis. Differences in lysis levels are evident between different strains and under divergent culture conditions (Nariya and Inouye, 2008). Lysis is suppressed in strains differentiating in the presence of prey bacteria (Berleman and Kirby, 2007). This is consistent with data indicating that sporulation can only be induced in populations exposed to the products of autolysis or other nutrients (Wireman, 1979). An additional advantage of autolysis may be the release of the important spore-coat-specific protein S that has no signal sequence (Dworkin, 1996).

DNA Dynamics

Unlike *B. subtilis*, *M. xanthus* differentiation is not linked to cell division, and therefore DNA dynamics in these bacteria could be subject to less precise regulation.

However, recent studies of *M. xanthus* DNA dynamics reveal that there is developmental regulation of chromosome copy number and a characteristic spatial organization of chromosomes within myxospores (Rosario and Singer, 2007; Tzeng and Singer, 2005; Tzeng *et al.*, 2006). Upon entry into stationary phase, *M. xanthus* chromosome copy number drops to one per cell, as might be expected in non-dividing cells (Tzeng and Singer, 2005). Myxospores, however, invariably contain two copies of the chromosome with both the origins and termini localized to the periphery of the cell. This duplication may be an advantage during germination allowing rapid gene expression of needed products (Tzeng and Singer, 2005). If DNA replication is blocked in the early stages of development, all development following aggregation is halted (Tzeng *et al.*, 2006). DNA replication inhibitors added 12 hours after initiation of development had no appreciable effect on development, indicating a window in which DNA replication is required for sporulation. Using a temperature-sensitive DnaB mutant, it was demonstrated that development can be stopped and restarted based on DNA replication status (Rosario and Singer, 2007). The mechanisms of chromosome segregation in *M. xanthus* have not been subject to investigation. The fate of the DNA from lysed cells also remains enigmatic, although there are reports of developmentally regulated DNases in this species (Martinez-Canamero *et al.*, 1991).

Streptomycetes

Members of the genus *Streptomyces* of the High G+C Gram positive phylum Actinobacteria are renowned for their production of secondary metabolites, including a variety of antibiotics. These bacteria specialize in the breakdown of refractory plant material and secrete a multitude of enzymes that control the health and viability of their soil competitors, both bacterial and fungal (Chater, 2006). Streptomycetes have an unusual developmental cycle that is composed of both vegetative growth and differentiation into spore-bearing stalks, or aerial hyphae (Chater, 1993). The

vegetative cells, or substrate mycelium, result from the germination of spores and can differentiate into hyphal stalks growing up out of the substrate. These mature to form long filaments composed of spores allowing dispersal, potentially through arthropod ingestion, to a more nutrient-rich environment (Chater, 2006).

Developmental Program

Streptomycetes utilize mycelial growth, spreading through a substrate and secreting degradative enzymes, until the nutrients are exhausted. The effectors of differentiation in the Streptomycetes vary considerably between different species as might be expected given the ancient origin of this lineage and the early divergence between species (Chater and Horinouchi, 2003). However, some commonalities exist such as the production of a γ-butyrolactone signal molecule that accumulates in the environment and, responding to cell density, participates in physiological and morphological differentiation. Also important for differentiation are production of the biosurfactant, SapB, which allows hyphal growth out of the surface, and the master regulator of differentiation, AdpA, which activates a signaling cascade involved in morphogenesis. Aerial hyphae are long filaments that extend into the air and subsequently septate and mature into spores.

Programmed Cell Death:

Although there is recent data on a PCD event very early in *Streptomyces* development, here we will focus on the PCD that coincides with sporulation (Manteca *et al.*, 2007). Because aerial hyphae by definition only grow on nutrient depleted media, the regulated lysis of cells comprising the substrate mycelium has been shown to provide essential nutrients to aid the development of the reproductive cell type (Mendez *et al.*, 1985; Miguelez *et al.*, 2000). This lysis is often concomitant with the secretion of a variety of antibiotics, ostensibly protecting the lysate from opportunistic ingestion by soil inhabitants. Cells within the substrate mycelium have two distinct pathways

towards cell death, either cell wall autolysis or physiological cell death (Miguelez *et al.*, 1999). A small minority of the mycelial cells experience rapid cell wall degradation concurrent with the initial formation of aerial hyphae, while most undergo an orderly process of cellular degeneration that coincides with the differentiation of aerial hyphae into spores. This process begins with the disorganization and degradation of the nucleoid and other cytoplasmic components (Miguelez *et al.*, 1999). Subsequently, the cells shrink and plasma membranes become permeable. The cell wall, however, remains intact, presumably to retain the integrity of colony structure providing aerial hyphae with mechanical support (Miguelez *et al.*, 2000).

DNA Dynamics

The Streptomycetes substrate mycelia grow by tip extension and septate only infrequently (Flardh, 2003). Mycelia are largely multinucleate cells and nucleoids are arranged along the length of the cell. Factors regulating DNA replication and the mechanism for this arrangement, termed nucleoid migration, remain obscure. Aerial mycelia form long multinucleate filaments as well, which undergo synchronous septation prior to spore development producing uninuclear spores (Flardh, 2003). Nucleoid segregation has been described in sporulating cells and appears to utilize homologues of the partitioning proteins ParA and ParB (Kim *et al.*, 2000). ParB binds to the origin-proximal *parS* sites and forms a nucleoprotein complex that has been shown in other bacteria to serve as an anchor for active segregation (Jakimowicz *et al.*, 2002). Streptomycetes examined thus far have linear chromosomes and telomere-like proteins that cap the ends of these chromosomes and aid complete replication, directly contributing to genetic stability (Hopwood, 2006). Although the exact mechanism controlling chromosome copy number during differentiation is unknown, there is a clear correlation between DNA replication and developmental stage (Smulczyk-Krawczyszyn *et al.*, 2006). Deletion of a high-affinity DnaA box far from the *oriC* in

the *Streptomyces coelicolor* chromosome resulted in more frequent chromosome replication and accelerated formation of aerial mycelia (Smulczyk-Krawczyszyn *et al.*, 2006). Conversely, addition of extra DnaA boxes of this type led to slow growth of substrate mycelia and significantly delayed sporulation. These results indicate a tight coordination of DNA replication with differentiation in the Streptomycetes. Much of the mycelial DNA is fated for degradation (Miguelez *et al.*, 1999). DNA degradation had two peaks during Streptomycetes development, upon initial formation of aerial hyphae and upon initiation of sporulation. Nucleases that appear responsible for both periods of regulated destruction of the mycelial DNA have been characterized and appear to be developmentally regulated (Fernandez and Sanchez, 2002).

Conclusion

Despite distinct phylogeny, formation of resistant cells adapted for dispersal is common to all these bacteria. In order to complete differentiation, a cellular division of labor is induced through cell-to-cell signaling. This division of labor is correlated in the examples above with the relative positions of the cells involved. Disparate fates of these cells coincide with distinct transcriptional programs. In some cases, such as the S protein in *Myxococcus* and spore coat proteins in *Bacillus* and *Clostridium*, specific products are required by the dispersive cell type that can only be delivered by terminally differentiated cells. A more general pattern of cell lysis to provide nutrients for sporulation may also be at play.

Examples of bacterial differentiation that requires nutrients from lysed cells have been described as cannibalism, fratricide, and parasitism (Chater, 2006; Claverys and Havarstein, 2007). However, in the cases examined above, it may also be useful to consider the paradigm of programmed cell death, with its implied cooperation, to develop hypotheses about mechanisms and evolutionary origin (Ameisen, 2002). The cooperation seen in programmed cell death in bacteria may be characterized as “kin

selection" (Sachs *et al.*, 2004). Cooperation benefiting those of a common ancestor differs from other models of cooperation in that there is no pressure for a direct benefit for all parties involved. Hence this model of cooperation fits well with observations from bacterial programmed cell death where lysing cells derive no direct benefit from cellular suicide.

Important questions remain unanswered in the newly emerging field of bacterial programmed cell death. In *Myxococcus*, preliminary investigations have defined some of the parameters that select for and against sociality (Velicer and Stredwick, 2002). However, much work remains to be done, especially in the *Bacillus* and Clostridial lineages. In these organisms, the biological consequences of a failure to undergo PCD have not been examined, especially as regards overall fitness in environments that are less defined than typical laboratory growth. Competition assays between cells with and without the ability to undergo PCD are required to clarify the conditions that select for programmed cell death in a variety of environments. Although no true homologues of cell death regulators from eukaryotes, the caspases, have been confirmed as of this writing, several bacterial groups have caspase-like cell death effectors (Rice and Bayles, 2008). The plant pathogen *Xanthomonas campestris* produces a caspase-like protein during nutrition stress that cross-reacts with antibodies to human caspase. Cyanobacteria of the genus *Trichodesmium* produce a caspase-like protein upon entry into death phase. This protein can be inhibited by a caspase-specific inhibitor and crossreacts with polyclonal antibodies to human caspase-3. Further investigation into these bacterial caspase-like proteins may reveal important information about the evolution of programmed cell death. However, the presence of proteins with significant homology to eukaryotic caspases remains the exception in the bacterial world, not the rule (Rice and Bayles, 2008).

The discovery of developmentally regulated DNases in diverse lineages suggests that mechanisms for utilization of the DNA of the terminally differentiated cells have been selected over the course of evolution. However, none of the work in this area has demonstrated subsequent uptake of the DNA fragments by neighboring cells. Detailed understanding of the molecular mechanism behind the progressive cellular degradation of the cell membrane and cell wall seen in these examples is also lacking. Further studies on these systems could contribute to our understanding of bacterial differentiation, the investigations of bacterial multicellularity, and evolutionary models of cooperation. Finally, given the centrality of PCD in eukaryotic development and disease, the potential impact of new, simpler models for this process would be significant (Ameisen, 2002; Golstein *et al.*, 2003).

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CHAPTER 2

CYTOLOGY OF TERMINALLY DIFFERENTIATED *EPULOPISCIUM* MOTHER CELLS

Abstract

Cell death is a necessary part of development for many organisms, including some bacteria. *Epulopiscium* sp. Type B, a member of the Firmicutes, is a large (up to 300 μm), cigar-shaped bacterial symbiont of surgeonfish that propagates itself by forming multiple intracellular offspring. This unusual form of reproduction is an apparent modification of a developmental program used by some Firmicutes to produce an endospore. At the onset of offspring formation, the *Epulopiscium* cell divides at both poles. The polar cells are engulfed by the larger mother cell and grow within it. At the final stages of development, the *Epulopiscium* mother cell lyses. Here we describe changes in *Epulopiscium* cell structure throughout the developmental cycle, focusing on mother-cell DNA replication and cell death. DNA replication was examined by labeling cells with the nucleotide analogue bromodeoxyuridine. As expected, DNA replication occurs in the developing offspring. However, well after passage of genetic information from parent to offspring is complete, DNA within the mother cell continues to replicate. Using fluorescence microscopy, we found that near the end of the offspring growth cycle, mother-cell DNA disintegrates. The mother-cell membrane and wall deteriorate as well. DNA replication within this terminally differentiated cell suggests the importance of mother-cell nucleoids in cell maintenance and the development of offspring. Cytological examination revealed hallmarks of programmed cell death in the *Epulopiscium* mother cell late in the developmental cycle. The programmed death of the mother cell maintains the metabolic activity of this large cell and may also allow for the timely release of

resources accumulated in the mother cell to provide nutrients to populations of these intestinal microbes and their host.

Introduction

Programmed cell death (PCD) is central to multicellular life (Ameisen, 2002). The incredible morphological complexity of some eukaryotes is facilitated by PCD during embryogenesis (Qu *et al.*, 2007). Careful regulation of cellular self-destruction is important for homeostasis, and PCD malfunction is linked to severe diseases in multicellular organisms (Feig and Peter, 2007). Given the significance of the appropriate timing and regulation of cell death, there has been a recent emphasis on exploring alternative PCD model systems, and expanding conceptual frameworks to include microorganisms (Ameisen, 2002; Golstein *et al.*, 2003). On the surface, evolution of PCD in unicellular life forms is counterintuitive; cellular "suicide" would limit the ability to pass along genetic material and therefore be subject to negative selection. Nevertheless, examples of PCD in single-celled organisms abound (Ameisen, 2002; Engelberg-Kulka *et al.*, 2006; Golstein *et al.*, 2003; Lewis, 2000). One theme linking unicellular and multicellular forms of PCD is sociality (Ameisen, 2002). For example, the slime mold *Dictyostelium discoideum* undergoes a predictable cell death program as part of its response to starvation (Levraud *et al.*, 2003). The normally unicellular, amoeboid soil microorganism forms multicellular aggregates that develop into fruiting bodies with dormant spores at the top. The stalk of the fruiting body is composed of cells that have undergone autophagic vacuolar PCD (Golstein *et al.*, 2003; Levraud *et al.*, 2003). It has been postulated that dying stalk cells may also provide nutrients to developing spores. Thus, *Dictyostelium* has evolved the capability to preserve the population genome through differentiation into resistant spores, at the cost of the genetic resources of a portion of the aggregate.

Endospore formation in the Firmicutes (low G+C Gram-positive bacteria) provides an example of a bacterial developmental program employing PCD. The death and lysis of the mother cell during sporulation has been best characterized in the model organism *Bacillus subtilis* (Errington, 2003; Hosoya *et al.*, 2007). In response to nutrient deprivation and high cell density, *B. subtilis* initiates the formation of an endospore (Errington, 2003). The process begins with a modified form of cell division. Instead of creating two equivalent vegetative daughter cells by binary fission, the sporulating cell divides asymmetrically forming a forespore and mother cell. The smaller forespore is subsequently engulfed by the mother cell. Each of these two separate cellular compartments contains a chromosome and the two cells follow divergent transcriptional programs that are essential for proper endospore maturation. Mother cell lysis in *B. subtilis* is carried out by a group of autolysins that are under the control of σ^K, the final transcriptional regulator active in the mother cell during sporulation (Hosoya *et al.*, 2007). Mother cell lysis is advantageous for *B. subtilis* as it removes an obstacle to endospore germination and outgrowth. Additionally, the PCD of the mother cell releases nutrients that may aid other sporulating cells of the population in completing this energetically expensive differentiation program (Lewis, 2000; Veening *et al.*, 2008).

Epulopiscium sp. Type B is an unusually large (~250 μm x 50 μm) bacterium, and also a member of the Firmicutes (Angert *et al.*, 1993). This gastrointestinal symbiont of the tropical reef surgeonfish *Naso tonganus* is not yet in culture. Based on their natural environment and preliminary genomic data, *Epulopiscium* Type B populations are believed to play a role in breakdown of carbohydrates from the algae that form the bulk of the host's diet (Clements and Choat, 1995). Binary fission has never been observed in *Epulopiscium* Type B, instead these cells form multiple internal progeny (see Figure 1). *Epulopiscium* offspring development follows a fairly predictable cycle

correlated with the daily feeding pattern of its host (Angert and Clements, 2004; Montgomery and Pollak, 1988). This leads to developmentally synchronized *Epulopiscium* populations. Vivipary in *Epulopiscium* shares major morphological features and stages with endospore formation (Angert *et al.*, 1996; Angert and Clements, 2004; Flint *et al.*, 2005), although the process is modified. At the onset of offspring initiation, the *Epulopiscium* cell divides at both poles. DNA is packaged inside these polar compartments, which are subsequently engulfed by the large mother

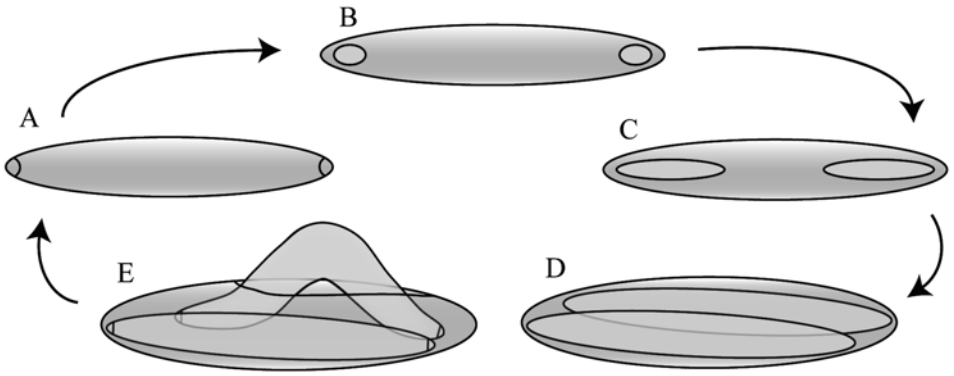


Figure 1. *Epulopiscium* sp. Type B life cycle. (A) Polar offspring are engulfed by the larger mother cell. (B) Fully engulfed offspring (C) grow within the mother cell until they (D) nearly fill the mother-cell cytoplasm. (E) Finally, "mature" offspring cells emerge from the mother-cell envelope. Note that prior to emergence, offspring cells begin to develop the next generation of offspring by dividing at their poles.

cell. The fully engulfed offspring grow to fill the mother-cell cytoplasm. The offspring initiate the next round of polar division while still encased in the mother cell. Eventually, the mother cell lyses, releasing the offspring. As seen in the models of

PCD, *Epulopiscium* uses, as a normal part of its life cycle, the lysis of one cell type (the mother cell) to facilitate the development of another (the offspring). *Epulopiscium* cells are distinguished also by the remarkable quantity and location of their cellular DNA. In the mother cell, DNA is arranged in a mesh-like network at the periphery of the cytoplasm (Robinow and Angert, 1998). This DNA is tightly associated with the highly infolded cell membrane (Fishelson *et al.*, 1985; Robinow and Angert, 1998). The large quantity of DNA in *Epulopiscium* cells is composed of tens of thousands of copies of the *Epulopiscium* genome (Mendell *et al.*, 2008). Polyploidy in *Epulopiscium* is likely an adaptation to large cell size and may permit transcription of any gene at disparate locations within the cell. In addition, genome copy number corresponds to cell size, suggesting a link between DNA replication dynamics and developmental stage.

To explore DNA replication dynamics with respect to *Epulopiscium* development, we labeled cells with the nucleotide analogue bromodeoxyuridine (BrdUrd). This method is routine for detecting active bacteria in environmental samples (Artursson *et al.*, 2005; Pernthaler *et al.*, 2002; Urbach *et al.*, 1999; Walters and Field, 2006), and has also been used to investigate the location of newly replicated DNA within bacteria during differentiation and development (Lewis and Errington, 1997; Ward and Angert, 2008). We verified that replication occurs inside the developing offspring. In addition, we found evidence of replication of mother-cell DNA in *Epulopiscium* cells that contained growing offspring. This was unexpected given that the mother cell had already produced all of its offspring cells and no more mother-cell DNA would be directly inherited. We also present a cytological description of changes in the *Epulopiscium* mother cell throughout development. As the mother cell ages, the regular net-like arrangement of DNA and its associate membrane is less apparent, compared to younger cells. Cell wall staining revealed disintegration of mother-cell

peptidoglycan late in development. These observations demonstrate that DNA replication occurs in terminally differentiated mother cells. In addition, our observations support the idea that the *Epulopiscium* mother cell undergoes PCD.

Materials and Methods

In Situ *Bromodeoxyuridine Labeling of* Epulopiscium

Naso tonganus was collected by spear fishing from outer reefs near Lizard Island, Great Barrier Reef, Australia. The fish intestinal tract was removed and placed inside a portable anaerobic bag (I2R Model X-27-27, Instruments for Research and Industry, Cheltenham, PA) that had been purged of oxygen and filled with CO_2 several hours in advance. After introduction of the sample, the bag was resealed and a GasPak (Hydrogen + CO_2, BBL Gas Pack System, Becton Dickinson Co., Cockeysville, MD) placed within the bag was activated.

BrdUrd labeling was performed using previously described methods (Ward and Angert, 2008), except incubations took place at temperatures comparable to the local water temperature (~24°C). Briefly, BrdUrd and thymidine were injected into sections of the intestinal tract that had been cordoned off with cotton string. The final concentrations in the experimental samples were approximately 40 μM BrdUrd and 30 nM thymidine, while control samples were 40 μM thymidine. Subsamples were taken from the intestinal lumen every hour by cutting open and removing the contents of one of the cordoned-off sections of intestine. Labeling took place over the course of three hours, and subsamples were fixed in paraformaldehyde (1% final concentration, EM 2 Grade, Electron Microscopy Sciences, Hatfield, PA) for 1 hour at room temperature. Fixed samples were washed twice in phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 1 L deionized H_2O, pH 8), suspended in 1:1 PBS:ethanol and stored at -20°C. Samples were shipped to the laboratory and stored in a -20°C freezer until processed. Unless otherwise stated, chemicals and

enzymes used in these studies were purchased from Sigma-Aldrich Co., Saint Louis, MO.

Immunodetection of BrdUrd in Epulopiscium *Cells*

Wells of approximately 2 x 1 cm were hand-drawn onto microscope slides (Corning, Corning, NY) using the hydrophobic PAP Pen (Daido Sangyo Co., Tokyo, Japan). These wells were treated with 0.1% poly-L lysine, for 3 min, then the solution was removed by aspiration, and the wells rinsed with deionized water. A sample of labeled *Epulopiscium* cells was washed three times with GTE (50 mM glucose; 25 mM Tris-Cl, pH 8; 10 mM EDTA). Each time, cells were pelleted by centrifugation at 3,000 G (Eppendorf Micro Centrifuge 5451 C, Westbury, NY) to remove smaller debris. Cells were resuspended in GTE, placed in the poly-lysine coated wells and incubated 4 min at room temp. Wells were aspirated and allowed to air dry completely. Cells were permeabilized by incubation in a lysis solution (5 mg/ml lysozyme, 2 mg/ml achromopeptidase, 2,000 U/ml mutanolysin) for one hour at 37°C in a humid chamber. Detection of BrdUrd was carried out using strand breakage induction by photolysis (SBIP) (Li and Darzynkiewicz, 1995). Sample processing was as previously described (Ward and Angert, 2008), with the following minor modifications. The optimal UV irradiation time was 2 min. In order to reduce autofluorescence, after washing the secondary antibody from the slides, cells were incubated in trypan blue (0.25 mg/ml, pH 4.4) for 3 min and washed twice with PBS. Coverslips were mounted in 1:1 Citifluor (Ted Pella, Inc., Redding, CA) and PBS and 0.02 mg/ml 4',6-diamidino-2-phenylindole (DAPI). Cells that were examined for membrane conformation were stained with 0.5 μM Mitotraker Red (Invitrogen, Carlsbad, CA) and trypan blue treatment was omitted. *Epulopiscium* cell wall material (N-acetylglucosamine) was stained with 1 μg/ml Cy3-conjugated wheat germ agglutinin (Invitrogen).

Phase-contrast, Nomarski DIC and fluorescence microscopy we were performed using an Olympus BX61 epifluorescence microscope, with 40x UPlanFl (N.A. 0.75) and 100x UPlanApo (N.A. 1.35) objectives. The microscope is equipped with filter cubes for viewing fluorescein, 7-aminoactinomycin D/Mitotracker Red, and DAPI fluorescence. Images were acquired using a Cooke SensiCam (Cooke Corp., Romulus, MI) and Slidebook software (Intelligent Imaging Inc., Denver, CO). Cell measurements were made using Slidebook and volume was calculated using the formula for a prolate ellipsoid ($V=4/3\pi ab^2$). Figures were assembled using Adobe Illustrator CS.

Results

Detection of Newly Replicated DNA in Epulopiscium *sp. cells*

In a previous study, we developed a protocol for labeling intestinal bacteria with BrdUrd (Ward and Angert, 2008). This approach, with minor modifications, was applied to intestinal microbial communities in *N. tonganus* that include the large bacterium *Epulopiscium* sp. Type B. For labeling *Epulopiscium* cells, BrdUrd was injected into the excised intestinal tract of the host and the intact intestine was incubated under anaerobic conditions. We know from previous observations that *Epulopiscium* cells remain alive and motile in the intestinal tract for at least four hours after capture and death of the host fish. Samples of intestinal contents were removed and fixed at one-hour intervals over the course of three hours. We labeled intestinal bacteria in one fish collected at 11 AM and a second fish collected at 4 PM. Large numbers of labeled *Epulopiscium* cells were recovered only from the fish caught at 11 AM and so those cells are described here. No significant difference was observed in the intensity and arrangement of the immunolocalization pattern of BrdUrd in *Epulopiscium* cells from the one, two, and three-hour incubations (data not shown).

We therefore focused our efforts on characterizing the localization patterns of BrdUrd within *Epulopiscium* cells incubated for 1 hour.

The BrdUrd labeled cells in this study comprised a population of *Epulopiscium* that was in the process of offspring elongation (see Figure 1). On average, offspring occupied 9.4% of mother-cell volume. Average lengths and widths of mother cells and offspring, and average numbers of offspring are reported in Table 1. By

Table 1. Average lengths and widths of *Epulopiscium* sp. Type B mother cells and offspring, and average number of offspring.

Mother Cell Length	**Mother Cell Width**	**Offspring Number**	**Offspring Length**	**Offspring Width**
150 +/- 16[c]	39 +/- 6[c]	1.84[c]	135 +/- 17[c]	22 +/- 3[c]
122 +/- 23[a]	34 +/- 7[a]	2.3[a]	49 +/- 13[a]	10 +/- 2[a]
215.2 ± 39.77[b]	34.8 ± 6.22[b]	2[b]	7.4 ± 1.1[b]	2.7 ± 0.61[b]

a: BrdU-positive cells, n=56, this study, b: Angert and Clements, 2004, c: Unlabeled cells, n=50, this study
All measurements are averages expressed in microns

comparison, average mother-cell volumes occupied by offspring calculated from other populations at different developmental stages ranged from 0.01% to 78%. The labeled *Epulopiscium* offspring were therefore in the relatively early, but not initial, stages of development. Within this window in the *Epulopiscium* life cycle, the mother cell is terminally differentiated, and will produce no additional offspring. We detected foci of immunolocalized BrdUrd, representing newly replicated DNA, in approximately 60% of the *Epulopiscium* population. Prior to BrdUrd detection, these cells were indistinguisible from other *Epulopiscium* populations with respect to cytology (data not shown).

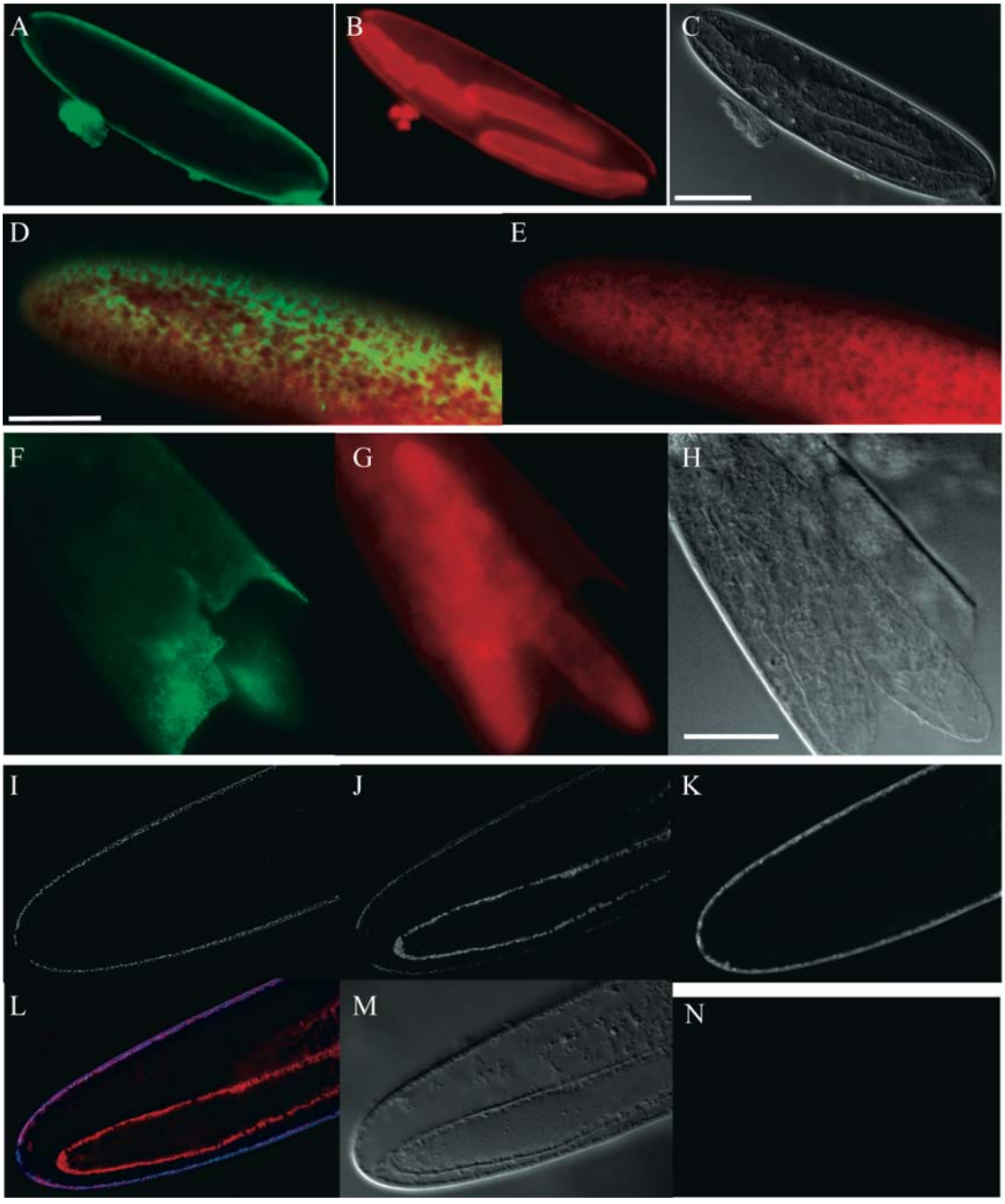
Spatial Arrangement of New DNA within the Mother Cell

In *Epulopiscium* cells, the newly replicated DNA labeled with BrdUrd co-localized with offspring and mother-cell total DNA (Figure 2). Most of the BrdUrd signal (shown in green) was located in the mother cell. At this stage of development, the mother-cell DNA (Figure 2E, shown in red) forms a distinct, mesh-like network at the periphery of the cytoplasm and the foci of BrdUrd co-localized with the DNA network. BrdUrd localization appeared less intense in the offspring and was best visualized in exposed offspring cells, i.e. in cells in which the mother-cell envelope was torn away during processing (Figure 2F-H). Other than the differences in overall mother cell and offspring labeling, regional patterns of BrdUrd accumulation were not generally observed.

We explored the location of newly replicated DNA with respect to the membrane system using a fluorescent membrane stain on the BrdUrd-labeled samples (Figure 2 I – M). The bulk of the DNA forms a layer just inside the membrane, and some string-like projections of DNA are found deeper in the cytoplasm. The membrane itself appears interspersed with the DNA. The mother-cell membrane staining was variable in intensity, which could be interpreted as areas of greater and lesser amounts of membrane invaginations in the plane under examination (Figure 2K). This would be consistent with a three dimensional network of membrane associated with the DNA mesh, where membrane invaginations project into gaps in the DNA network. No obvious bias in sites of BrdUrd labeling with respect to the membrane or location within a cell were observed. None of the thymidine-labeled control samples processed in parallel showed any of the characteristic foci that represent incorporated BrdUrd (Figure 2N).

Figure 2.

DNA Replication in *Epulopiscium* sp. Type B. BrdUrd was immunolocalized (shown in green in panels A, D, F, and L, also shown in panel I) in a sample of *Epulopiscium* cells with elongated intracellular offspring. (A - C) An overview of a mother cell with three offspring cells. (A) BrdUrd localized most strongly at the periphery of the mother-cell cytoplasm, co-localizing with the mother-cell DNA (B). (C) Differential interference contrast (DIC) image. (D - E) Higher magnification images of a focal plane just under the surface of a mother cell, demonstrate that BrdUrd-labeled DNA corresponds to the mesh-like network of DNA. (F - H) A mother cell that was torn during processing reveals BrdUrd incorporation within offspring. (F) BrdUrd co-localizes with (G) offspring DNA. (H) DIC image illustrates the boundaries of the offspring cells. (I - M) Location of BrdUrd incorporation and total DNA relative to cell membrane. An overlay of (I) BrdUrd localization, (J) DNA and (K) membranes are shown in panel L. (M) DIC image illustrates the relative positions of these subcellular layers in relationship to the cell envelope. The thymidine-labeled control (N) cells processed to immunolocalize BrdUrd showed none of the characteristic foci representing BrdUrd incorporation into newly replicated DNA. Scale bars represent 20 μm (A - C) and 10 μm (D - N).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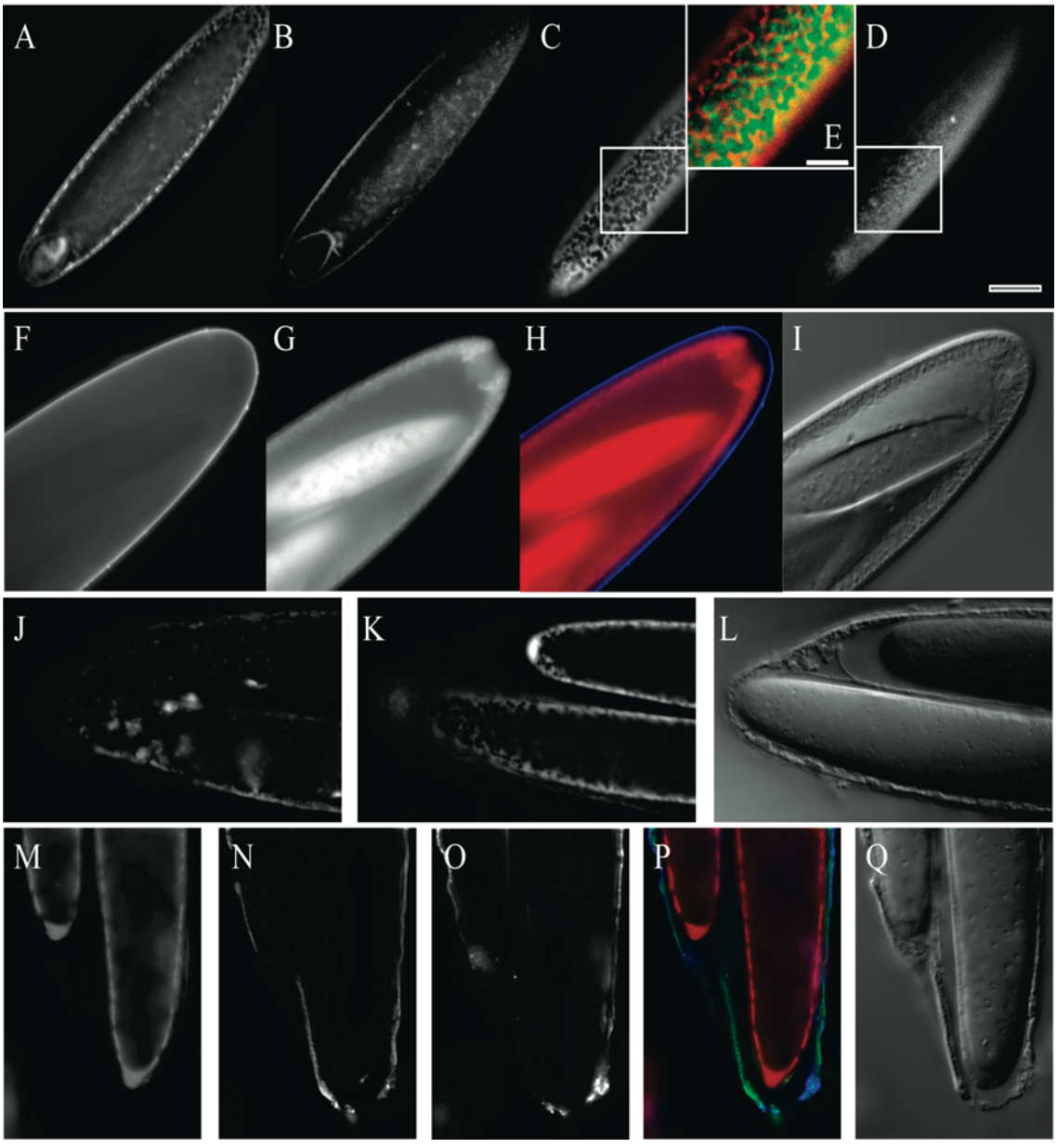
Characterization of Epulopiscium *sp. Cytology with Respect to Development*

DNA replication in the terminally differentiated *Epulopiscium* mother cells was unexpected. To place this observation in the broader context of the reproductive cycle, we performed a cytological characterization of cells fixed at various stages of development. Since *Epulopiscium* reproduction follows a 24-hour cycle (Angert and Clements, 2004; Montgomery and Pollak, 1988), we characterized populations of cells fixed at various times of the day to observe cells at a range of developmental stages. Populations in the early stages of offspring development contained some cells that had recently emerged from their mother cell and also "mature" offspring that were still retained (data not shown). Newly emerged cells showed a very clear spatial organization of DNA at the periphery of the cytoplasm (Figure 3A-E). The reticulate sheet formed by the DNA of these new mother cells stained intensely with DNA dyes. The DNA of small, fully engulfed offspring stained strongly as well, but this DNA filled almost the entire cellular compartment. The mother-cell membrane showed a pattern complementary to the mesh network of DNA with areas of high and lower intensity fluorescence. Upon overlay, a pattern emerged revealing that the areas of highest intensity of membrane staining coincide with the areas devoid of DNA (Figure3E). To examine the possibility that this mesh network was composed of invaginations in the cell wall as well, we used fluorescently-labeled wheat germ agglutinin to stain a component of the bacterial cell wall, N-acetylglucosamine. Cell wall material formed a smooth unbroken peripheral layer corresponding with the outer edge of the cell, which was positioned well outside the peripheral mesh of DNA. Late in the developmental cycle, offspring almost completely fill the *Epulopiscium* mother cell (Figure 3 J - Q). In this sample, the offspring had begun a new round of reproduction as evidenced by the condensed DNA near the tips of offspring cells

Figure 3.

Cytology of *Epulopiscium* sp. Type B Cell Development. This series of panels shows cells that represent various stages of the *Epulopiscium* life cycle. In overlay images (panels E, H and P), DNA is shown in red, membrane in green and cell wall in blue. (A - E) Newly emerged cells. These fluorescent micrographs show two different focal planes (medial in A and B, just beneath the mother-cell surface in C - E) of the same cell. The mother-cell DNA is located at the periphery of the cytoplasm (C) in a mesh-like network beneath a layer of membrane (D). These layers appear highly ordered and interlaced (E) in young mother cells, however, these complexes do not appear interlaced in offspring at this stage. (F - I) The mother cell, at an intermediate stage of development, shows the clear mesh pattern of DNA (G), a well-developed membrane layer (Figure 2 K) and a thick, regular cell wall with no invaginations (F and H, merge). (J - L) Mother cells, late in development, show an irregular, often patchy, membrane layer (J) and weak DNA staining (K) relative to their offspring. Offspring cells now have a distinct network of DNA. (M - Q) In stages immediately prior to offspring release, little DNA is visible in the mother-cell compartment (M) while both the membrane (N) and the cell wall (O) appear irregular and mottled (P, merge). Scale bar E, 5 μm, all other panels, 10 μm.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(Angert and Clements, 2004). The offspring contained a peripheral mesh-like network of DNA. Only faint staining of mother-cell DNA was seen at the periphery of the cytoplasm, and this DNA lacked the regularity and apparent organization found in mother cells at earlier stages of development. The membrane of the mother cell appeared fragmented and discontinuous and also considerably thickened compared to the offspring membrane. The mother-cell membrane exhibited some pockets of intense staining which could represent vesicles formed during disintegration. The mother-cell wall appeared irregular and blotchy, especially compared to the cells at earlier stages of development. A merged image of a cell stained for DNA, membrane and cell wall (Figure 3P) and its accompanying DIC image (Figure 3Q) illustrate the degeneration of the mother cell. Comparison of the cytology of these cells with earlier stages of development reveals a stark difference in levels of cellular integrity.

Discussion

Bacterial PCD systems are highly regulated genetic programs enabling cellular suicide (Lewis, 2000; Rice and Bayles, 2008). The phylogenetic diversity of organisms that employ PCD reveals the broad need for regulated self-destruction programs in the bacterial world. An analogy has been made between PCD in multicellular organisms and aggregate or colonial forms of bacteria such as the Myxobacteria (members of the Deltaproteobacteria) and Actinobacteria (Ameisen, 2002). Through cell-to-cell communications, social control is exerted on a population of these bacteria such that some cells are fated to die while others serve to pass on genetic information (Dworkin, 1996). For both of these bacterial models, differentiation is brought on by nutrient depletion. Resistant spores are formed atop a stalk-like structure. PCD facilitates formation of structural supports and may provide nutrients that aid in differentiation (Fernandez and Sanchez, 2002; Mendez *et al.*, 1985; Miguelez *et al.*, 2000; Nariya and Inouye, 2008; Nicieza *et al.*, 1999).

PCD is employed in survival mechanisms of Firmicutes where nutrient depletion and crowding activate endospore formation. Recent genetic and cytological investigations into PCD of sporulating *B. subtilis* have begun to characterize this process (Hosoya *et al.*, 2007). Once the endospore matures, the mother cell undergoes a sequential deterioration, beginning with membrane disruption. Next, the developmentally regulated extracellular DNase NucB leaks into the mother cell and facilitates the breakdown of mother-cell DNA. In the final stages of mother cell demise, activities of autolysins CwlB, CwlC and CwlH dismantle the mother-cell wall, thus completing the process. During sporulation *B. subtilis* may be viewed as a transiently multicellular organism, a sporangium comprised of two cells (Ameisen, 2002). The mother cell takes on a somatic role, supporting development of the spore but unable to pass on its genetic material to offspring. The forespore is in essence the germline.

Another endospore-forming member of the Firmicutes that appears to undergo a similar process of mother-cell death is *Metabacterium polyspora*. Although capable of binary fission for a short period of its life cycle, this guinea pig gastrointestinal symbiont forms multiple endospores as its primary means of propagation. To accommodate the production of multiple viable spores, the basic developmental program has been modified substantially (Angert and Losick, 1998; Ward and Angert, 2008). In *B. subtilis,* replication initiation is suppressed during sporulation (Castilla-Llorente *et al.*, 2006), while in *M. polyspora* DNA replication continues throughout sporulation and primarily occurs in developing forespores (Ward and Angert, 2008). DNA replication within the forespores allows *M. polyspora* to accumulate genome copies to support formation of multiple forespores, and perhaps load endospores with multiple copies of the genome. Late in sporulation, the mother cell appears to pass through a sequential degradation process similar to that observed in *B. subtilis*. We have observed that, in *M. polyspora*, the mother-cell nucleoids collapse and

disintegrate prior to mother cell lysis, and the mother-cell wall becomes less resistant to muramidases (Ward and Angert, unpublished).

Although closely related to *M. polyspora*, replication dynamics and the developmental process in *Epulopiscium* sp. Type B appear to be further modified. Unlike *M. polyspora*, binary fission has never been observed in *Epulopiscium* sp. Type B. Instead, these cells rely solely on the formation of multiple intracellular offspring for reproduction. The observed accumulation of newly replicated DNA within the *Epulopiscium* mother cell was in marked contrast to our previous observations of replication in *M. polyspora*. This mother-cell DNA would never be directly passed on to offspring, which suggests that mother-cell DNA serves a somatic role; it is required for the development of the offspring, possibly by maintaining the metabolic capacity of the mother cell throughout the growth cycle. The degree of BrdUrd incorporation into mother-cell DNA may have limited the amount of BrdUrd that reached the offspring cytoplasm. Therefore we cannot draw any conclusions about relative differences in replication observed in mother and offspring cells. Late in the developmental cycle, as offspring mature and the mother cell ages, the mother-cell DNA becomes less apparent and eventually disappears. Concurrent with DNA disappearance, we observed the disruption of the mother-cell membrane and peptidoglycan wall. The cytological examination of *Epulopiscium* throughout development suggests that the mother cell undergoes PCD (Figure 4).

PCD as part of differentiation appears to be common among the endospore-forming Firmicutes. In the examples described above, modifications to replication patterns have evolved to suit the needs of some Firmicutes with unusual reproductive programs. In *B. subtilis*, DNA replication is shut off as the cell commits to sporulation (Castilla-Llorente *et al.*, 2006). Genes expressed in the mother cell throughout spore development are essential for endospore maturation. In addition, the appropriate

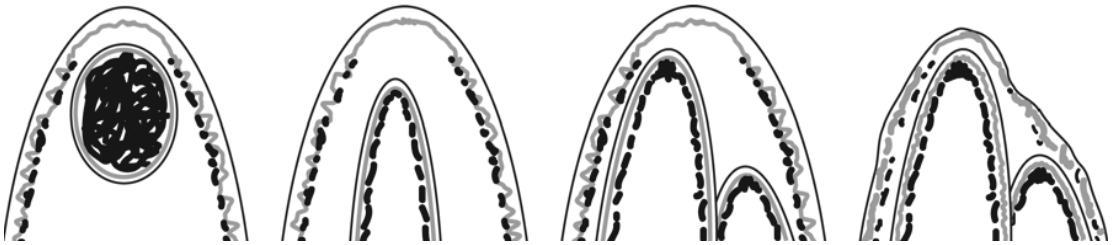

Figure 4. Model of Developmental Changes in the *Epulopiscium* sp. Type B Mother Cell. Shown are cross-sectional views of the pole of an *Epulopiscium* mother cell. In this illustration cell wall is shown in black, membranes in light gray and DNA in dark gray. (A) Early in offspring development, the mother-cell envelope and wall are rigid and well defined. Beneath the cell wall is the highly invaginated membrane system. Beneath this, is the mesh-like network of DNA. Early in development, offspring are closely associated with the pole. (B - C) As the offspring grow, DNA replication occurs within both the offspring and mother cell. (D) Late in the developmental cycle, the mother cell exhibits signs of PCD. The mother-cell DNA becomes less apparent and eventually disappears. The mother-cell membrane system and peptidoglycan wall deteriorate as well.

expression and activity of a number of genes under the control of the late stage mother-cell sigma factor are necessary for PCD. The *M. polyspora* mother cell likewise must contribute to spore development. In contrast, DNA replication is required during development of these spores so that the cell can produce enough genome copies to support the formation of multiple spores and the rapid cycling into sporulation immediately after endospore germination (Ward and Angert, 2008). For *Epulopiscium*, DNA replication in the mother cell and offspring is observed. Although it is an expensive investment, DNA replication in the mother cell is probably imperative to support the metabolic needs of this large bacterium and its developing internal offspring. The previously demonstrated correlation between cell size and

genome copy number in this bacterium further supports this conclusion (Mendell *et al.*, 2008).

A question that remains is the fate of the nutrients released during mother cell death. Nutrient release will be substantial due to three factors, the large size of the endosymbionts, their high density in the host intestinal tract and the daily cycle of offspring formation. Also, the *N. tonganus* gastonintestinal tract is a nuclease rich environment, ensuring easy utilization of DNA in particular (K. Clements, personal communication). An attractive hypothesis for the significance of release of large quanitities of somatic DNA is that it provides a nutrient storage molecule that may be taken up by the offspring or other mother cells in the population. The storage and timed release of phosphate may be particularly advantageous in a phosphate-limited marine environment (Karasov and Martinez Del Rio, 2007). However, the volume of nutrients released during this process suggests that other factors should be considered. Nutrient outputs would not be quarantined by *Epulopiscium*. It is possible that significant amounts may be scavenged by the host fish through assimilation of material released from lysed mother cells. This observation also focuses attention on the need to understand the role of the gut environment and its microbial community in selecting for the unique properties of *Epulopiscium*, large size, high mobility, vivipary and PCD.

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Kendal Clements and Howard Choat both contributed to this work, both with fish collection and interpretation of results.

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CHAPTER 3

DNA REPLICATION DURING ENDOSPORE DEVELOPMENT IN *METABACTERIUM POLYSPORA*

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Abstract

The guinea pig intestinal symbiont *Metabacterium polyspora* is an uncultured, endospore-forming member of the Firmicutes. Unlike most endospore-forming bacteria, sporulation is an obligate part of the *M. polyspora* life cycle when it is associated with a guinea pig. Binary fission is limited to a brief period in its life cycle, if exhibited at all. Instead, *M. polyspora* relies on the formation of multiple endospores for reproduction. Sporulation is initiated in most cells immediately after germination, which leaves little time for the cell to accumulate resources to support spore formation. Using immunolocalization of the nucleotide analogue bromodeoxyuridine (BrdU), we were able to follow replication dynamics in *M. polyspora*. BrdU was provided to cells within the guinea pig intestinal tract. The BrdU was incorporated into DNA located within the forespores and mother cell throughout development, at all stages prior to spore maturation. We found that within a sporangium, BrdU accumulation in developing forespores outpaced accumulation in the mother cell. Our results suggest that in *M. polyspora,* DNA replication is not suppressed during sporulation as it is in other endospore-forming bacteria. Replication within forespores would allow *M. polyspora* to maximize its reproductive potential and supply each endospore with at least one complete copy of the genome.

Introduction

In response to harsh environmental conditions, certain members of the Firmicutes produce a highly resistant and dormant cell, an endospore. This process allows the bacterium to preserve its genetic material while it waits for conditions to improve. For

a few lineages, endospore formation has further evolved into a means of propagation (Angert, 2005). *Metabacterium polyspora*, a gastrointestinal (GI) symbiont of guinea pigs, regularly produces multiple (up to 9) endospores (Robinow, 1951). Binary fission is restricted to a short period in the *M. polyspora* life cycle, if exhibited at all. The production of multiple endospores also helps *M. polyspora* sustain its symbiotic association. Mature endospores of *M. polyspora* rely on being consumed by their host to gain entry into the GI tract. Reproductive progression is coordinated with passage of *M. polyspora* through the GI tract. Consequently, most cells in a particular region of the GI tract share a similar stage of development. As resilient endospores, these anaerobes may survive the external, aerobic environment and the mechanical, chemical and enzymatic assaults faced in the upper GI tract better than vegetative cells.

Typical endospore formation, as exemplified by the model organism *Bacillus subtilis* (Errington, 2003; Hilbert and Piggot, 2004), begins with nucleoid remodeling; the chromosome replicates and forms an axial filament that spans the long axis of the cell (Ryter, 1965). The two replication origins are sequestered to opposite cell poles (Ben-Yehuda *et al.*, 2003; Wu and Errington, 2003), and initiation of subsequent rounds of DNA replication is suppressed (Castilla-Llorente *et al.*, 2006; Fujita and Losick, 2005). In a modification of normal bacterial cell division (Bi *et al.*, 1991), FtsZ rings form near both poles of the cell. Division occurs at only one of these sites and the other FtsZ ring eventually depolymerizes. This asymmetric division produces a single forespore and the larger mother cell. During division, the origin-proximal third of one chromosome is captured within the forespore. As development proceeds, the remainder of the trapped chromosome is transferred into the forespore. Once DNA translocation is complete, the forespore is engulfed by the mother cell. Through coordinated genetic programs within the two cells, the forespore is prepared for

dormancy and matures within the mother cell. Upon exposure to an environment favorable for growth, germinating spores resume normal DNA replication and chromosome segregation (Lewis and Errington, 1997).

Previous work has demonstrated that *M. polyspora* shares with *B. subtilis* many of the major morphological features and stages of endospore formation (Chatton and Perard,

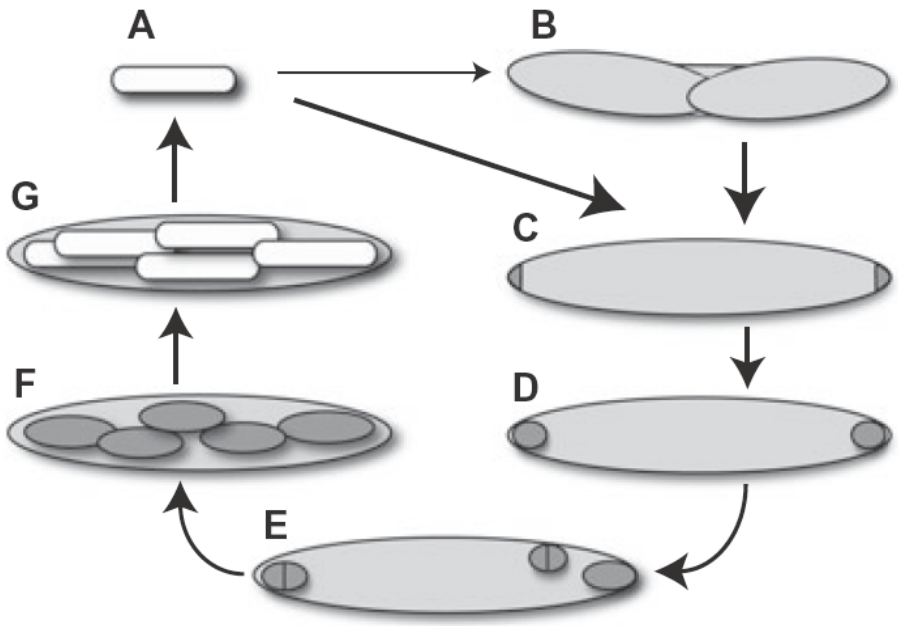


Figure 1. The life cycle of *M. polyspora*. Within the small intestine of its host, the guinea pig, *M. polyspora* endospores (A) germinate. A small proportion of the population undergoes binary fission (B), while most cells immediately undergo bipolar division (C). These polar forespores are engulfed (D), as the cells move with the intestinal contents into the ceacum. Fully engulfed forespores may undergo fission and are capable of migration within the sporangium (E). The forespores then elongate (F) prior to spore maturation (G) and release. Upon re-ingestion by the host, dormant endospores pass through the upper GI tract and then germinate to repeat the life cycle.

1913; Stunkel *et al.*, 1993). Modifications that allow for the production of multiple endospores have been observed (see Figure 1). Like *B. subtilis*, *M. polyspora* forms FtsZ rings at the poles of a sporulating cell but *M. polyspora* typically divides at both poles (Angert and Losick, 1998). DNA is trapped and packaged into the two forespores formed during bipolar division, while some DNA remains in the mother cell. Both forespores are then engulfed by the mother cell. The engulfed forespores can undergo additional rounds of division, also mediated by FtsZ. In this case, a Z ring forms at a medial position and the forespore divides into two roughly equal halves. Thus *M. polyspora* can form multiple forespores (generally 2 – 5) that mature into endospores. Although nucleoid dynamics throughout the *M. polyspora* life cycle have been documented (Angert and Losick, 1998), the details of genome copy number and replication timing are not known.

For *M. polyspora*, ensuring that each of its endospores contains a complete genome requires modification of the model sporulation program at the level of DNA replication and/or nucleoid segregation. In one possible scenario, all genome copies destined for the developing spores are produced prior to asymmetric division, and multiple copies are segregated to the poles at the start of sporulation. Alternatively, one or a few copies are segregated to each pole of the cell and partitioned into the forespores. After engulfment, the DNA in each forespore is replicated to generate the genetic material needed to support the development of multiple viable spores. Both of these proposed pathways present challenges for the cell. If replication is completed prior to the start of sporulation, segregation of multiple chromosomes could be mechanistically difficult (Egan *et al.*, 2005), and the number of viable progeny would be determined early in development. For the alternative scenario, rounds of replication would need to take place during sporulation, inside the developing forespores.

To investigate the timing and site of DNA replication with respect to development in *M. polyspora*, we have utilized immunolocalization of the nucleotide analogue bromodeoxyuridine (BrdU). Through this approach, we found a relationship between forespore formation and the site of BrdU accumulation, with engulfed forespores consistently providing a strong BrdU localization signal. These data suggest that *M. polyspora* allows replication of its DNA within developing forespores to ensure that all of its endospore progeny obtain a complete genetic complement.

Materials and Methods

Bacterial strains and growth conditions

Clostridium lentocellum ATCC 49066 was grown anaerobically at 38°C. Cultures were grown in a modified cellulose mineral salts (MCMS) medium (Tammali *et al.*, 2003) containing 11 mM KH_2PO_4, 11.5 mM K_2HPO_4, 33 mM urea, 6.6 mM $MgSO_4$, 1.4 mM $CaCl_2$, 12 mM sodium citrate, 0.9 mM cysteine, 66 μM $FeSO_4$, 5 g/L yeast extract, 2 g/L peptone, 0.1 % resazurin, supplemented with sucrose (1% w/v) just prior to inoculation. The medium was prepared in stoppered serum bottles with CO_2 headspace using the Hungate Method (Hungate, 1969). Strain SØ103, a low-thymine-requiring *Escherichia coli* K-12 derivative (Munch-Petersen and Neuhard, 1964), was obtained from the *E. coli* Genetic Stock Center (CGSC #5898) and grown at 37°C in Luria Burtani medium. Samples containing *Metabacterium polyspora* were collected from guinea pigs, *Cavia porcellus*, as described below. Procedures involving guinea pigs were designed under the guidance of the Cornell Center of Animal Resources and Education, and the Institutional Committee for Animal Care approved all protocols.

Bromodeoxyuridine Labeling of Bacteria in Culture

Labeling experiments were based on previously described methods (Lewis and Errington, 1997; Pernthaler *et al.*, 2002; Scupham, 2007; Urbach *et al.*, 1999). Growing cultures of *C. lentocellum* were supplemented with 40 μm

bromodeoxyuridine (BrdU) plus 33 nM thymidine. Cultures grown in media supplemented with thymidine only were used as negative controls. 10X stock solutions of BrdU and/or thymidine were freshly prepared in anaerobic MCMS for *C. lentocellum*, and added to exponentially growing cultures at an optical density of ~0.5 (3.5 hr after inoculation of 0.5 ml into 10 ml fresh media). Samples were removed from each serum vial every hour, starting at time 0. For DNA extraction, samples were fixed in 80% ethanol buffered with PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 1 L deionized H_2O, pH 8). For microscopy, cells were fixed by adding paraformaldehyde (1% final concentration, EM Grade, Electron Microscopy Sciences) to the media. Cells were immediately pelleted with centrifugation (10 min, 5000 G, 4°C) and resuspended in a solution of PBS and 1% paraformaldehyde. Cells were fixed for 2 hr, 4°C, then pelleted, washed once with PBS, and resuspended in a solution of 1:1 ethanol:PBS and stored at -20°C. For *E. coli* SØ103, BrdU and thymidine solutions were prepared in sterile water and exponentially growing cultures were labeled as described above.

Pulse-labeling of M. polyspora *with BrdU.*

Pulse-labeling of *M. polyspora* was accomplished within the guinea pig intestinal tract, which was excised from the body after euthanasia. The intestinal tract was divided into sections by tying off portions with cotton string. The volume of intestinal contents in each segment was estimated and 40 μM BrdU plus 33 nM thymidine or 40 μM thymidine (final concentrations), prepared in anaerobic MCMS, was injected into the intestinal sections. The sections were gently massaged to disperse the injected fluid. Samples were placed in plastic bags, purged with CO_2 to reduce oxygen exposure, and incubated at 40°C in a hybridization oven (FisherBiotech). Subsamples were taken every hour and fixed as described above, at room temperature for 30 min, then refrigerated at 4°C until prepared for long-term storage. For storage, samples

were diluted in PBS, filtered through loosely packed glass wool to remove large particulate matter, and stored in 1:1 ethanol:PBS at -20°C.

Continuous Labeling of M. polyspora in vivo *with BrdU*

To label *M. polyspora in vivo,* guinea pigs were provided drinking water containing either BrdU with thymidine or thymidine only. Animals were given ad libitum access to drinking water containing 2 mg/ml BrdU plus 3.3 μM thymidine for 2 days followed by 3 mg/ml BrdU with 5 μg/ml thymidine for 2 days. A control animal was given water containing 3.3 μM thymidine. At the end of the treatment period, animals were euthanized, dissected and intestinal samples immediately collected and fixed as described above.

Dot Blot analysis of BrdU incorporation

As a qualitative assessment of BrdU incorporation, DNA isolated from *C. lentocellum* and *E. coli* was subjected to immunomagnetic precipitation and dot blot analysis as previously described (Urbach *et al.*, 1999) with modifications to hybridization conditions. Salmon sperm DNA (1.25 mg/ml in PBS) was boiled for 1 min, frozen in a dry ice-ethanol bath, thawed, mixed 9:1 with mouse monoclonal anti-BrdU antibodies (Sigma, diluted 1:10 in PBS), and incubated for 30 min at room temp. Genomic DNA samples from *E. coli* or *C. lentocellum* were boiled for 1 min, frozen in dry ice-ethanol, thawed, mixed with 100 μl of the salmon sperm DNA-antibody mixture, and incubated for 30 min. Goat anti-mouse IgG-coated paramagnetic beads (Dynal Biotech) were washed twice in PBS and a 25 μl volume of beads was added to each sample, incubated for 30 min and washed seven times with 0.5 ml of PBS-BSA (1 mg/ml). BrdU-containing DNA was eluted by adding 25 μl of 1.7 mM BrdU (in PBS) and incubating for 30 min with constant agitation.

The DNA recovered from the magnetic beads was then subjected to dot blot analysis as follows. Sample DNA (1 μl of *E.coli* DNA or 3 μl of *C. lentocellum* DNA) was

diluted in TE (total volume 10 µl), denatured by the addition of 1 µl of 2N NaOH, incubated for 10 min (22°C), and blotted onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech). Membrane processing was as in Urbach *et al.* (1999) with modifications; DIG High Prime DNA Labeling and Detection Kit blocking solution (Roche) was used instead of PBS:BSA, and glycerol was omitted from the hybridizations. Alkaline phosphatase-conjugated secondary antibodies (Roche) were used and detected colorimetrically as per manufacturer instructions.

In situ *detection of BrdU*

Wells of 10-well, multitest slides (ICN Biomedicals, Inc.) were treated with 0.1% poly-L lysine (Sigma), for 1 min, then the solution was removed by aspiration, and the wells rinsed with deionized water. Cells washed with GTE (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8) were resuspended in GTE with lysozyme (2 mg/ml for *C. lentocellum*), then placed in a slide well and incubated 1 min at room temperature. Wells were aspirated, washed two times with PBS, and allowed to air dry completely. Each sample well was incubated in 4 N HCl for 1 hour at room temp while the slide was kept in a moist chamber. The slide wells were washed twice with PBS and incubated in DIG High Prime DNA Labeling and Detection Kit Blocking Reagent for 3 min at room temp. Affixed cells were subsequently incubated overnight (4°C) in monoclonal anti-BrdU (~1.5 mg/mL, clone BU-33, purified IgG, buffered aqueous, Sigma) diluted 1:1000 in DIG blocking solution. The slides were washed ten times in PBS and incubated in secondary antibody [FITC-conjugated anti-mouse IgG (Jackson Immunoresearch) with 0.02 mg/ml 4',6-diamidino-2-phenylindole (DAPI)] in DIG blocking solution for an hour at room temp. Slides were washed ten times with PBS. Coverslips were mounted in 1:1 Citifluor (Ted Pella, Inc.) plus deionized H_2O, and DAPI.

Strand Break Induction by Photolysis (SBIP) was used for BrdU signal amplification (Li and Darzynkiewicz, 1995). Guinea pig intestinal samples were washed in GTE and affixed to 10-well slides prepared as described above. Cells were permeabilized by one of the following treatments: cells were incubated in proteinase K (Sigma, 1 mg/ml, 55°C, 1 hour) or cells were incubated in achromopeptidase (Sigma) 5 mg/ml in GTE, pH 7, 37°C for 1 hour followed by mutanolysin (Sigma) 10,000 U/ml in PBS, 37°C for 2 hours, and lysozyme (Sigma) 5 mg/ml in GTE, pH 7, 37°C for 1 hour. SBIP was carried out according to Li *et al*. (1995) with minor modifications. Cells affixed to a slide were placed on a transilluminator and exposed to UV light for 20 to 40 seconds (Fisher Biotech Electrophoresis Systems, 312 nm variable intensity) and then incubated in terminal deoxynucleotidyl transferase (TdT) end-labeling solution (Fermentas, Hanover, MD) with 250 U/ml TdT and 5 nM BrdUTP (Sigma). Cells were washed 10 times with PBS, and incubated in antibody solutions as described above, with the substitution of either Hoechst 33342 (0.01 mg/ml) or 7-amino-actinomycin D (7-AAD, 10 μg/ml) for the DNA stain DAPI. Cells with two and three hour BrdU/thymidine incubations were analyzed as above except for primary antibody concentration (1:500). Coverslips were mounted with SlowFade (Molecular Probes) and sealed to prevent dehydration.

The guinea pigs used for *in vivo* labeling contained sparse populations of *M. polyspora*. To increase the recovery and retention of *M. polyspora*, the immunolocalization protocol was modified as follows. Cell permeabilization, SBIP and TdT incubations were carried out in a microcentrifuge tube. Cells were permeabilized by a combined treatment of achromopeptidase, lysozyme and mutanolysin at concentrations listed above, in GTE, but the samples were incubated for 45 min at 37°C. For SBIP, samples were labeled using the direct detection method instead of immunodetection (Li *et al.*, 1996). Immunodetection of BrdU is the more

sensitive detection method but the direct labeling method provides faster sample processing. In our hands, both methods produced identical results. After exposure to UV light, cells were incubated with 2.5 nm Chromotide BODIPY FL-14-dUTP and 250 µl of TdT end-labeling solution for 1 hour at 37°C. Cells were washed ten times with PBS. DNA was stained with 50 µm Syto 9 (Invitrogen) for 15 min. The cells were washed with GTE, applied to poly-L-lysine treated slides and coverslips mounted in Slowfade.

Phase-contrast, Nomarski DIC and fluorescence microscopy were performed using an Olympus BX61 epifluorescent microscope, with 40x UPlanFl (N.A. 0.75) and 100x UPlanApo (N.A. 1.35) objectives. The microscope is equipped with filter cubes for viewing fluorescein, 7-AAD, and DAPI/Hoechst fluorescence. Images were acquired using a Cooke SensiCam and Slidebook software (Intelligent Imaging Inc). All histograms of *M. polyspora* images were normalized to between 1700 and 2300. Exposure time for all *C. lentocellum* images was 2 seconds and histograms were adjusted to identical parameters. Figures were assembled using Adobe Illustrator CS. To determine the mean fluorescence (pixel) intensity of forespores or mother cells, micrographs were deconvolved first to eliminate fluorescence from out-of-focus fluorescent objects (Slidebook, Intelligent Imaging). The forespore and mother-cell compartments were delineated using Slidebook's draw tool and mean pixel intensity of each was determined. Volume of each forespore was calculated using the formula for a prolate ellipsoid ($Vp = 4/3\pi ab^2$). Ratios of forespore to mother cell mean pixel intensities were determined and plotted relative to the forespore volume in Microsoft Excel. Statistical analysis was performed in Microsoft Excel (regression).

Results

Optimization of BrdU labeling protocols in Clostridium lentocellum

BrdU is taken up and incorporated into the DNA of many, but not all, growing wild-type bacteria (Pernthaler *et al.*, 2002; Urbach *et al.*, 1999; Vestereng and Kovacs, 2004). In *Escherichia coli* and *B. subtilis*, efficient incorporation is seen in strains in which the endogenous thymidylate synthetase gene, *thyA*, has been mutated (Frisch and Visser, 1960; Yoshikawa, 1967). Since *M. polyspora* is not available in culture, and cannot be genetically manipulated, we began by assaying the ability of a close relative, *C. lentocellum*, to incorporate BrdU into its genomic DNA. We followed an approach developed by Urbach *et al*. (1999) using BrdU to label actively growing bacteria in cultures and microcosms of environmental samples. Urbach and others have found that adding a small amount of thymidine during BrdU labeling experiments enhances BrdU incorporation. The presence of exogenous thymidine presumably suppresses endogenous synthesis of thymidine by inhibiting the activity of thymidylate synthetase .

Various concentrations of BrdU, ranging from 20 μm to 40 μm, were added to cultures of *C. lentocellum* at the time of inoculation, or during exponential phase in growing cultures. The cultures were monitored by following change in optical density at 600 nm (data not shown). The highest concentration of BrdU that showed no effect on growth rate was 40 μm, so this concentration was used for subsequent experiments. Total genomic DNA was extracted from samples taken from BrdU or thymidine-treated control cultures at 30, 60 and 90 min after addition of the nucleotide. The DNA was then subjected to immunomagnetic separation using monoclonal antibodies specific for BrdU. This procedure enriches for BrdU-containing DNA but cannot eliminate all unlabeled DNA from the sample (Urbach *et al.*, 1999). DNA dot blots were used to assess BrdU assimilation. DNA taken from the *E. coli* thymine

auxotroph SØ103 showed good incorporation of BrdU after only 30 min. Incorporation of BrdU in *C. lentocellum* genomic DNA was visible after 30 min of growth in BrdU-containing media and increased with length of incubation (data not shown). These results demonstrated that *C. lentocellum* cells could incorporate BrdU into their DNA.

We then developed protocols for *in situ* detection of BrdU in intact *C. lentocellum* cells. Since BrdU has been used as a mutagen, albeit at higher concentrations than those used in our labeling protocol, *in situ* detection allowed us to verify that BrdU-containing cells had normal nucleoid morphology and exhibited no obvious cell cycle anomalies. BrdU co-localized with the nucleoids of cells grown in the presence of the thymidine analogue (Figure 2). The BrdU localization was seen as multiple, discrete foci within the cell. Thymidine control cells and cells exposed to BrdU appeared similar, both in morphology and in nucleoid conformation.

To enhance detection of BrdU, we investigated the use of a signal amplification method in *C. lentocellum* cells. Strand Break Induction by Photolysis (SBIP) takes advantage of the photolabile nature of DNA that contains BrdU to create additional targets for BrdU-tri-phosphate (BrdUTP) incorporation and immunodetection (Li and Darzynkiewicz, 1995; Li *et al.*, 1995). When exposed to UV light, the major class of lesion formed in BrdU-DNA is single-strand breaks in the sugar-phosphate backbone (Kondrat'ev and Skavronskaya, 1971). Subsequent incubation in the presence of the enzyme terminal deoxynucleotidyl transferase (TdT) and BrdUTP drives the addition of BrdU onto 3' hydroxyls of the broken DNA. This increase in the number of BrdU targets leads to an increase in signal (Figure 2).

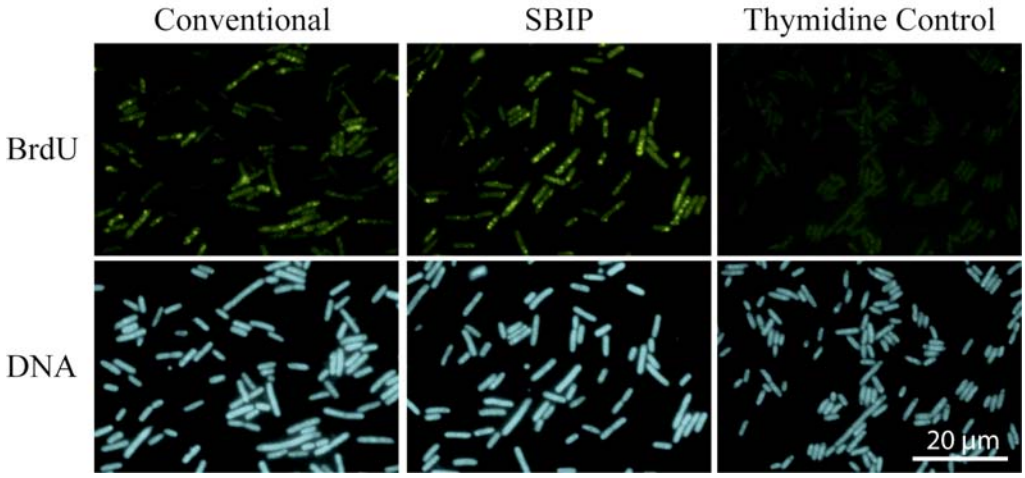


Figure 2. Localization of BrdU in *C. lentocellum*. BrdU incorporation in *C. lentocellum*, a close relative of *M. polyspora*, was verified using conventional immunodetection techniques. Strand Breakage Induction by Photolysis (SBIP), a signal amplification method, yielded a visible increase in signal intensity. The thymidine control revealed no BrdU-immunolocalization signal. All panels are shown at the same magnification and have the same exposure time and histogram parameters. Scale bar represents 20 μm.

Immunodetection of BrdU in pulse-labeled M. polyspora *cells*

As *M. polyspora* is not currently in culture, BrdU labeling of live cells was conducted *ex situ,* within the intestinal tract of its host after the guinea pig was euthanized. We chose this approach because we knew that *M. polyspora* cells remain viable within the GI tract for several hours after the host has been euthanized, and the method would be more broadly applicable to other experimental systems. In addition, it allowed us to label cells for defined periods of time and have more control over BrdU and thymidine concentrations in different GI tract environments. In all BrdU treatments, thymidine (at approximately 33 nm) was included. Samples were withdrawn from the intestinal lumen once every hour over the course of four hours of incubation. Distinct labeling patterns were most evident after four hours. Localization patterns from earlier time points, 2 or 3 hours, were considerably weaker but were consistent with four-hour observations (data not shown). Reproduction and development of endospores in *M. polyspora* is tied to passage of the bacterium through the host gastrointestinal tract (Angert and Losick, 1998). Cells of *M. polyspora* recovered from the small intestine were either phase-grey, germinating spores or cells after outgrowth, some of which had initiated sporulation. The ceacum samples were dominated by mother cells that harbored mature endospores, although cells at earlier stages of sporulation were also present. From these two locations, all stages of development were recovered.

Cells representing progressive stages in the *M. polyspora* life cycle were identified, photographed, and scored for their BrdU localization pattern (Figure 3). Cells were categorized into stages of development similar to the major developmental transitions observed in *B. subtilis* (Errington, 2003; Hilbert and Piggot, 2004). These include binary fission, polar division, complete forespore engulfment, forespore growth/maturation, and sporangia with mature, phase-bright endospores. The *M. polyspora* cells that were fixed while engaged in binary fission (n = 56) displayed

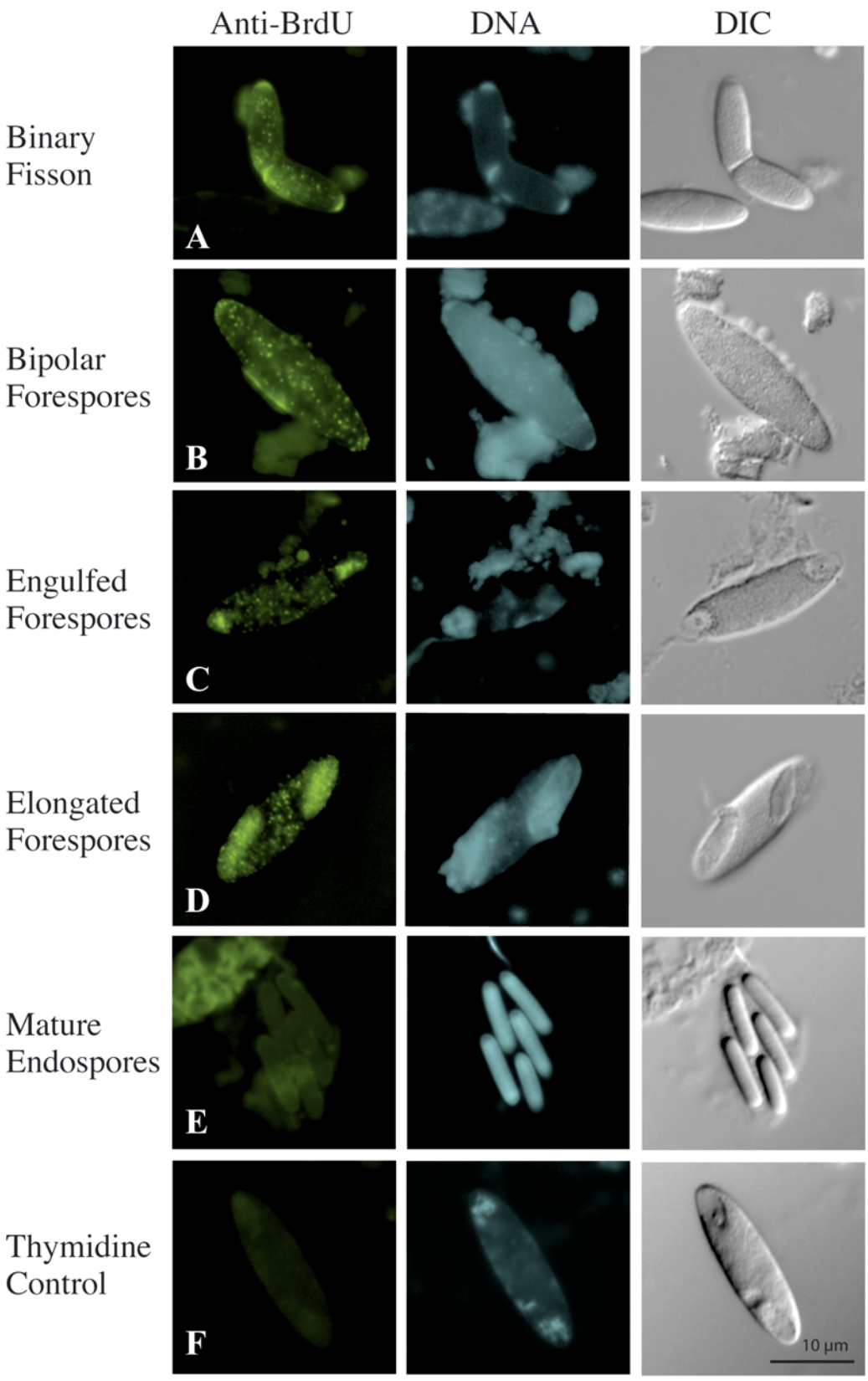
scattered BrdU foci throughout the cytoplasm and often contained more concentrated signal at the cell poles (Figure 3A). The polar BrdU signal colocalized with DNA inside the developing forespores. These forespores were discerned by the presence of polar septa visible by differential interference contrast (DIC) microscopy. More dispersed BrdU signal was found throughout the mother-cell cytoplasm, and was associated with the more diffuse mother-cell DNA.

In cells that were not undergoing binary fission (i.e. cells with no visible septum at midcell) and cells with only polar septa, bright foci representing BrdU were found throughout the cytoplasm (n = 74) (Figure 3B). Cells with polar septa also showed a concentration of signal at the poles, which colocalized with the brightest DNA staining.

For cells later in development, ones that contained two small engulfed, polar forespores (n = 40) (Figure 3C), BrdU localized as foci within the mother-cell cytoplasm. These mother-cell foci were generally less prevalent than those of the cells described above. The most conspicuous signal, however, was seen within the engulfed forespores, where BrdU foci were densely packed. Even within forespores that appeared uniformly fluorescent, deconvolution microscopy revealed distinct fluorescent foci (data not shown). Late in development, large, elongated forespores (n = 85) (Figure 3D) harbored bright, densely packed foci. BrdU foci were also found within the mother-cell cytoplasm. While not all cellular DNA contained BrdU, the BrdU signal intensity generally corresponded with the location and intensity of DNA staining. Little to no BrdU was detected in mother cells with mature, phase-bright endospores. BrdU was never seen in phase-bright endospores (Figure 3E). Like all endospores, the phase-bright endospores of *M. polyspora* are impermeable to large proteins and are resistant to lysis by muramidases (Nicholson *et al.*, 2000). The permeabilization treatments used in this study would not strip the coat off these mature

Figure 3. Localization of BrdU in *M. polyspora*.

Panels shown represent a survey of the major developmental stages in the *M. polyspora* life cycle. Immunolocalization of BrdU (left), location of DAPI or Hoechst staining (center) and Nomarski differential interference contrast (DIC, right) micrographs are shown for each field. Cells in panels (A – D) harbor distinct foci of BrdU within the mother-cell cytoplasm as well as within developing forespores. (A) Shown are the products of binary fission with strong BrdU signal localized to the poles. (B) A cell that has undergone bipolar division has a fairly uniform pattern of BrdU localization throughout the mother cell and incipient forespores. (C) This cell contains two fully engulfed forespores. (D) A cell later in development with two elongated forespores shows greater BrdU-associated fluorescence within the forespore cytoplasm as compared to the mother-cell cytoplasm. (E) This typical sporangium, with five mature endospores, shows no BrdU-associated signal because antibodies cannot enter. *In addition, DNA dyes cannot enter the cytoplasm of mature endospores, but instead stain the spore cortex. (F) Thymidine-incubated control shows no BrdU localization. All micrographs are the same magnification. Scale bar represents 10 μm.


Anti-BrdU
DNA
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Binary Fisson
A
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B
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C
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D
Mature Endospores
E
Thymidine Control
F
10 µm

spores or degrade the underlying cortex. As a result, we would not be able to detect BrdU within any mature endospores by immunolocalization. In addition, DNA dyes cannot enter the core of mature phase-bright endospores (Flint *et al.*, 2005; Setlow *et al.*, 2002). Instead, these dyes associate with the cortex of the spore (Setlow *et al.*, 2002), leading to a distinct peripheral staining pattern, similar to what we observed with mature endospores of *M. polyspora* (Figure 3E). Finally, the thymidine negative control showed no foci (Figure 3F).

Based on fluorescence intensity, it appeared that within a given sporangium, more BrdU was incorporated into the DNA within forespores than in the mother cell. It also appeared that BrdU accumulated in both the forespores and mother cell as the forespores grew. To further analyze this apparent trend, we compared the mean immunofluorescence intensity associated with the forespore and mother cell cytoplasm for twenty-seven sporangia with elongated forespores. These cells represented a range of forespore numbers (1 – 4), mean pixel intensities (22 – 591), mother-cell lengths (13.6 – 25.1 μm), and forespore-to-mother-cell-volume ratios (0.06 – 0.46). We confirmed that as mean fluorescence intensity in the mother cell increased, fluorescence in the forespores increased (Figure 4 A). Next, we charted the relative mean fluorescence intensity (that is, the ratio of forespore-to-mother-cell fluorescence) as a function of forespore volume. A positive correlation between the relative forespore fluorescence and forespore size was found (Figure 4B), The number of forespores within the mother cell did not appear to affect this trend. This result indicates that BrdU accumulates in forespores as they grow.

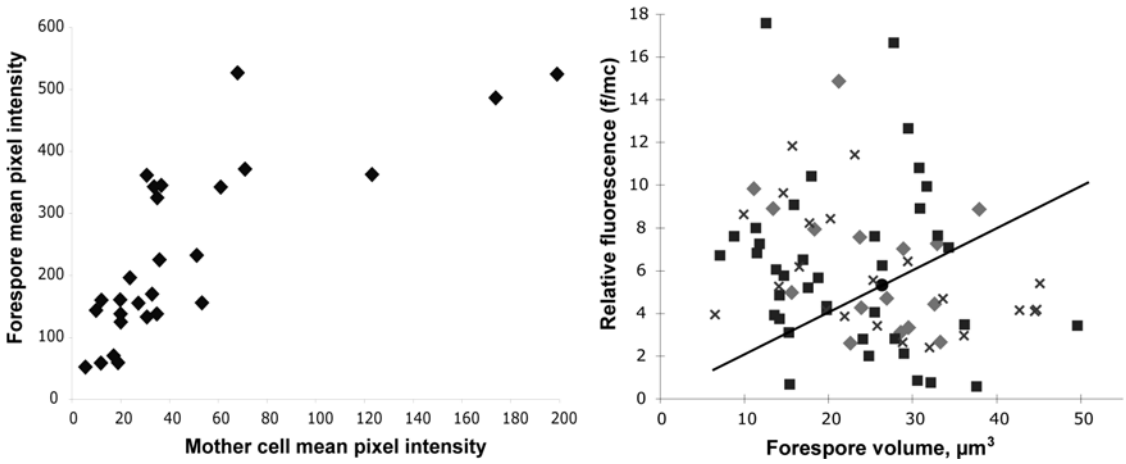

Figure 4. BrdU accumulates within a sporangium as development progresses. (A) The positive correlation between BrdU accumulation in the forespores and mother cell cytoplasm. For each sporangium, the mean fluorescence intensity within forespores was plotted against mean fluorescence found within the mother cell cytoplasm. (B) There is a positive correlation between the relative forespore-to-mother-cell fluorescence as forespores grow. Shown is a plot of the ratio of BrdU-associated mean fluorescence intensity in the cytoplasm of a forespore to the cytoplasm of its mother-cell (f/mc) compared with the cytoplasmic volume of the forespore. Symbols represent the number of forespores in each developing sporangium (one forespore, ●; two forespores, ◆; three forespores, ✖; four forespores, ■).

Validation of BrdU labeling patterns in M. polyspora

One concern with using an *ex situ* system to label anaerobic bacteria is that the bacteria are not in an entirely natural state, and injection of BrdU into the intestinal tract could introduce oxidants into the environment. Anaerobic bacteria incapable of neutralizing these oxidants may suffer DNA damage (Takeuchi *et al.*, 1999b). In such instances, BrdU could accumulate in DNA as a result of repair and not solely during replication. To validate the *ex situ* labeling results, *M. polyspora* cells were labeled *in vivo*, within live guinea pigs. We reasoned that under these conditions, BrdU incorporation should predominantly label sites of active replication. BrdU was

provided in the drinking water of guinea pigs for several days prior to the collection of intestinal samples. Immediately after euthanasia, the guinea pigs were dissected and intestinal contents were chemically fixed. From these samples, all stages of *M. polyspora* endospore development were observed (not shown). The pattern of BrdU immunolocalization was punctate and it colocalized with cellular DNA (Figure 5). Within a given sporangium, more BrdU accumulated in forespores than the mother cell. Since the pattern of accumulation was the same in the two different labeling conditions, we conclude that BrdU incorporation in *M. polyspora* cells is most likely due to incorporation of the nucleotide analogue during replication of DNA and not repair.

Discussion

Bromodeoxyuridine incorporation in cells in native ecosystems

The thymidine analogue BrdU has a long history as a tool for studying cell cycle in eukaryotes and bacteria (Coote and Binnie, 1986; Frisch and Visser, 1960; Hackett and Hanawalt, 1966; Leif *et al.*, 2004; Meselson and Stahl, 1958; Yoshikawa, 1967). Until recently, most of the experiments with bacteria have been performed under carefully controlled conditions, often using strains with defects in endogenous thymidine uptake and metabolism. Several studies have now demonstrated the ability of bacteria in natural settings to take up and utilize BrdU, and this method has been used to identify actively growing populations (Artursson *et al.*, 2005; Mou *et al.*, 2007; Pernthaler *et al.*, 2002; Scupham, 2007; Urbach *et al.*, 1999; Walters and Field, 2006). To the best of our knowledge, the findings presented here are the first time BrdU incorporation has been used to follow DNA replication with respect to development in an uncultured bacterium.

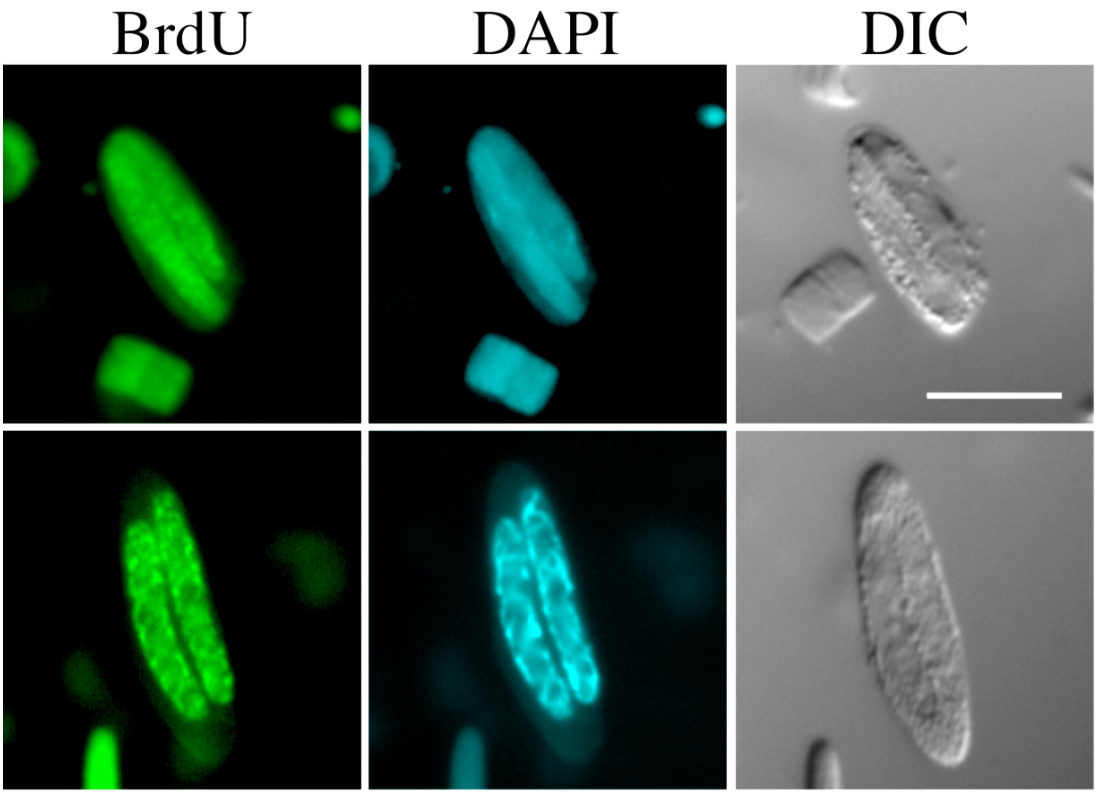

Figure 5. Localization of BrdU in *M. polyspora* labeled *in vivo*. Panels shown represent a cell with large forespores.

Preliminary experiments with *C. lentocellum* point out some of the limitations in using BrdU for cell cycle studies in wild-type bacteria. BrdU immunolocalization in *C. lentocellum* produced distinct foci distributed throughout the cell but with no discernable pattern, rather than the strong localized signal reported in pulse-labeling studies using a thymidine-requiring mutant of *B. subtilis* (Lewis and Errington, 1997). A possible cause of the punctuate pattern observed in *C. lentocellum, M. polyspora* and other bacteria (Zahradka *et al.*, 2006) is discontinuous incorporation of BrdU. Since thymidine kinase has a marked preference for thymidine over BrdU (Coote and Binnie, 1986; Hackett and Hanawalt, 1966), thymidine would be phosphorylated and incorporated into DNA during replication when it was available. In nutritionally complex, heterogeneous environments it is difficult to determine the availability of thymidine to a single cell. In our labeling protocol, we provide some exogenous thymidine to suppress *de novo* synthesis. Under these conditions cells would naturally take up and incorporate a mixture of BrdU and thymidine during replication, SBIP BrdU immunodetection was carefully optimized in the *M. polyspora* studies and care was taken to minimize DNA damage in these cells during *ex situ* incubations. Anaerobic bacteria incapable of neutralizing oxidants may suffer DNA damage upon exposure to oxygen (Keyer and Imlay, 1996; Takeuchi *et al.*, 1999a; Takeuchi *et al.*, 2000). Such DNA damage could be detected in SBIP assays but also it would be detected in the thymidine-incubated controls, similar to how DNA damage is detected with BrdUTP in apoptotic eukaryotic cells (Li and Darzynkiewicz, 1995; Li *et al.*, 1996). To confirm that the BrdU immunolocalization patterns observed in the *ex situ* studies were primarily due to incorporation of BrdU during replication and not repair, we performed continuous labeling of intestinal bacteria within live guinea pigs. This form of labeling is commonly used to identify proliferating cells in animals () and has recently been used to characterize active microbial populations in the intestinal tract of

turkeys (Scupham, 2007). When labeled *in vivo*, *M. polyspora* cells exhibited stage-specific patterns of BrdU accumulation that were essentially identical to those observed in the *ex situ* labeling experiments. BrdU localized to the mother cell and forespore DNA in a punctate pattern and it accumulated preferentially in the forespores. We would not expect that *M. polyspora* cells within the GI tract of a healthy host would require extensive DNA repair during its normal lifecycle. Therefore we conclude that BrdU accumulation, using either labeling protocol, represents sites of DNA replication.

DNA replication within developing sporangia

The patterns of BrdU localization reported here indicate that replication occurs in *M. polyspora* during the brief period of vegetative growth. Replication also occurs within the forespores throughout development. Finally, we noted that little or no replication occurs in the mother cell once endospores mature. With the incubation times needed to accumulate detectable levels of BrdU in pulse-labeled *M. polyspora* cells, it is difficult to determine the precise timing of replication with respect to stages of development. Therefore we focused on determining if BrdU was incorporated into forespore DNA after engulfment. By comparing homologous morphological transitions in *Bacillus* and *Clostridium* spp. we can determine some temporal boundaries. Under ideal conditions for *B. subtilis,* engulfment is completed 100 min after initiation of sporulation. After this time no more mother-cell-replicated DNA could reach the forespore. Mature phase-bright, heat-resistant spores are abundant by 7 hours after onset. The human intestinal pathogen *C. perfringens* sporulates almost as rapidly as *B. subtilis* (McClane, 1997). These rapid spore-formers provide a minimum time frame for morphological transitions. In many other clostridia, sporulation is much more protracted, taking days to complete (Alsaker and Papoutsakis, 2005; Arcuri *et al.*, 2000; Paredes *et al.*, 2005; Santangelo *et al.*, 1998).

Unfortunately, sporulation has been extensively studied only in members of the group I clostridia (Collins *et al.*, 1994), which are not closely related to *M. polyspora* (clostridium cluster XIVb). Its closest cultured relative, *C. lentocellum* (strain SG6, for example), requires 3 days of growth on cellulose before phase-bright endospores are detected (Tammali *et al.*, 2003).

Perhaps a comparison can be made with other close relatives of *M. polyspora*, namely the sporulating morphotypes of the *Epulopiscium*-like surgeonfish intestinal symbionts (Flint *et al.*, 2005). Morphotype C symbionts do not undergo binary fission and instead rely on the daily production of two endospores for reproduction. This process follows a predictable 24-hour cycle, leading to naturally synchronized populations with respect to spore development. Engulfed forespores are formed in as few as three hours after outgrowth, and forespore growth occurs over the next eleven hours (Flint *et al.*, 2005). Post-engulfment development takes 5 to 11 hours to complete. Endospore development in these cells is probably entrained by external signals. Therefore, developmental progression in these intestinal symbionts may be regulated and constrained by factors that do not come in to play during development in *M. polyspora*. If forespore development, including elongation, requires a similar length of time in *M. polyspora* as it does in other endospore-forming bacteria, the BrdU detected within the largest forespores would have accumulated after engulfment. These data suggest that the strong signal observed within the forespores was the result of synthesis, not simply packaging of DNA synthesized in the predivisional cell.

Conclusions

Replication initiation is tightly regulated in sporulating *B. subtilis*. As a cell begins to sporulate, only one round of chromosome replication may occur (Hilbert and Piggot, 2004). From engulfment through maturation of the endospore, no additional DNA synthesis takes place. This repression of replication initiation is affected by the

transcription factor Spo0A (Castilla-Llorente *et al.*, 2006; Fujita and Losick, 2005). *In vitro* studies revealed that activated Spo0A binds to sites near the *B. subtilis oriC*, which prevents DnaA-mediated unwinding of the replication origin (Castilla-Llorente *et al.*, 2006). If initiation were likewise repressed in *M. polyspora*, the predivisional cell would need to undergo multiple rounds of replication prior to asymmetric division. In *M. polyspora*, however, sporulation is initiated shortly after germination. This leaves little time for DNA replication and accumulation of resources. During asymmetric division, the capture of origins and segregation of multiple genomes to each rudimentary forespore could be logistically difficult (Egan *et al.*, 2005). Furthermore, DNA replication prior to sporulation, and subsequent repression during sporulation, would predetermine the reproductive potential of the cell at an early stage of its life cycle, perhaps even before reaching the nutrient-rich caecal environment. For these reasons, it seems more likely that a cell with the capacity to produce multiple offspring in the form of endospores would require genome replication during sporulation.

Our data supports the following model for replication during the life cycle of *M. polyspora*. DNA replication is initiated shortly after germination. The predivisional cell would only need two or three copies of the genome to begin sporulation, therefore asymmetric division may occur immediately. As the cell divides at both poles, DNA is segregated to each of the rudimentary forespores. After engulfment, the genome(s) within the forespores replicate, segregate, and the forespores may divide. Even after the number of offspring is set, replication continues so that the endospore is loaded with multiple genome copies.

Replication during sporulation allows *M. polyspora* to accumulate resources at times when they are available and abundant, that is, while *M. polyspora* is within the guinea pig ceacum. More importantly, the cell could produce offspring in response to the

carrying capacity of the environment. In addition, the ability to replicate DNA within the growing forespore would allow each offspring to accumulate the genome copies needed (at least two) for immediate sporulation after germination. This failsafe would enable a cell to form a single endospore even under conditions that could not support normal *M. polyspora* reproduction.

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CHAPTER 4

THE MICROBIAL COMMUNITY OF THE HINDGUT CHAMBER OF *POMACANTHUS SEXSTRIATUS*

Abstract:

The gastrointestinal bacterial symbionts of reef fishes have been implicated in their ability to digest refractory dietary components. *Pomacanthus sexstriatus*, the six-banded angelfish, is an omnivorous reef species found in the western Pacific Ocean, including the Great Barrier Reef, Australia. The microbial community in the hindgut chamber of *P. sexstriatus* was investigated through phylogenetic analysis of the 16S rRNA gene and fluorescent *in situ* hybridization. Almost half of the sequences obtained were most closely related to sequences from bacterial lineages known for specialization in fermentation (Bacteroidetes and Firmicutes). More remarkable, however, was the presence of a considerable (29%) number of sequences that cluster among those of the sulfur-reducing bacteria within the orders *Desulfovibrionales* and *Desulfobacterales.* Archaeal diversity was also examined and 16S rDNA sequences related to the *Thermoplasmales* genus were recovered. These sequences were most similar to sequences of putative methanogens from diverse anoxic environments. The unusual prevalence of sequences affiliated with sulfur-reducing bacteria suggests there may be a physiological need for sulfur metabolism in the intestinal tracts of *P. sexstriatus,* in addition to the more standard fermentative community. This gastrointestinal consortium may facilitate an expanded dietary range for *P. sexstriatus*.

Introduction:

It is becoming increasingly apparent that complex communities of gastrointestinal symbionts are a feature of marine herbivorous fishes as well as terrestrial vertebrate herbivores (Karasov and Martinez Del Rio, 2007). Work in terrestrial herbivores has shown the importance of these communities, especially bacteria capable of

fermentation, in breaking down carbohydrates into short-chain fatty acids that are more easily absorbed by the host (Karasov and Martinez Del Rio, 2007). Investigation into the intestinal microbiota in herbivorous fish have shown similar dynamics (Choat and Clements, 1998). Short-chain fatty acid levels in the posterior intestine of fishes are generally high in fish species with a differentiated hindgut chamber, but high values are also known in species that lack obvious anatomical specialization of the intestine (Clements and Choat, 1995). A variety of dietary components are thought to serve as fermentation substrates in these environments (Choat and Clements, 1998). These dietary components include the flora and fauna of coral reef ecosystems, highlighting the importance of coral reef fish, and their digestive microbiota, in this delicate and rich environments (Choat and Clements, 1998; Hixon, 1991).

Characterization of microbial communities is complicated by the inability to culture the majority of bacteria using standard methods (Amann *et al.*, 1995). One of the most common culture-independent approaches used to characterize the microbiota of a complex ecosystem is to extract total DNA from an environmental sample, amplify the small subunit rRNA genes and characterize these sequences to infer phylogenetic affiliation and microbial diversity (Amann *et al.*, 1995). This method has greatly expanded our understanding of microbial ecology and diversity, providing insights no other methods could (DeLong and Pace, 2001). However, phylogenetic surveys using the 16S rRNA genes are subject to biases which may skew a 16S RNA gene library relative to the diversity in the environmental sample (Forney *et al.*, 2004). To address this issue, fluorescent *in situ* hybridization or other quantitive methods may be used to verify the proportions of targeted groups of bacterial cells (Amann *et al.*, 1995).

In this study, the microbial community from the hindgut of the omnivorous coral reef fish *P. sexstriatus* was analyzed through sequencing a 16S rDNA gene clone library. This species of fish was chosen for investigation because it has a broad-based diet of

both algal and animal material (Bellwood et al. 2004; Mantyka & Bellwood 2007), thus raising the possibility of an unusually diverse flora, and because it has a hindgut chamber, a feature that has been associated with high levels of fermentation in other species (Clements & Raubenheimer 2006). The majority of the resulting 228 non-chimeric sequences obtained in this study were affiliated with the phyla *Bacteroidetes* (29.2%), *Firmicutes (*19.8%) and Proteobacteria, more specifically the order *Desulfovibrionales (*29.6%). Fluorescent *in situ* hybridization results confirmed that members of the order *Desulfovibrionales* constituted a significant portion of the community (34%).

The methanogen functional gene *mcrA* was amplified from the sample and an Archaeal 16S rDNA gene clone library was constructed. All Archaeal sequences obtained were distantly affiliated with the *Thermoplasmatales* group (Ruepp *et al.*, 2000; Schleper *et al.*, 1995), but the closest sequence similarities were found with clones obtained from other gastronintestinal microbial consortia (Boonapatcharoen *et al.*, 2007; Hara *et al.*, 2002; Huang *et al.*, 2003; Shinzato *et al.*, 1999; Shinzato *et al.*, 2001; Wright *et al.*, 2006; Wright *et al.*, 2007). Fluorescent *in situ* hybridization results confirmed that approximately 4% of the hindgut microbiota is affiliated with this group of Archaea.

The observed composition of the microbial community in *Pomacanthus sexstriatus* is unusual compared to other similar habitats (Clements *et al.*, 2006; Fidopiastis *et al.*, 2006; Mountfort *et al.*, 2002.). The community structure implies a dietary substrate for the large numbers of sulfur-reducing bacteria is present in the hindgut and identifies an unusual Archaeal group, possibly involved in methanogenesis . The data from this study may serve as a basis for further work on the role of microbial metabolism in carbon and sulfur cycling mediated by coral reef fish.

Materials and Methods

Sampling and DNA extraction

A single *Pomacanthus sexstriatus* was collected by spearfishing from outer reefs around Lizard Island, Great Barrier Reef, Australia. The contents of the hindgut chamber were fixed in 80% ethanol and stored at -20° C until processed. To extract DNA, cells from a hindgut sample were pelleted by centrifugation and washed in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and subjected to lysis at 37°C for one hour (final concentrations 5% sodium dodecyl sulfate, 3.3 mg/ml lysozyme, and 0.4 mg/ml proteinase K in TE). Sodium chloride (final 0.75 M) and Cetyl trimethylammonium brominde (CTAB, 1%) were added and the sample was incubated at 65°C for 10 min. Phenol: chloroform extractions and an ethanol precipitation were followed by re-suspension of the community DNA sample in TE. Unless otherwise stated, chemicals were purchased from Sigma.

Bacterial 16S rRNA Gene Clone Library Construction

PCR was performed on a 1:100 dilution of the community DNA sample to amplify the 16S rDNA using primers 8F and 1492R (Lane, 1991) using the following thermocycle regime: 95°C 15 min, 25X 95°C, 30 s, 47°C, 30 s, 72°C, 2 min. HotStarTaq polymerase (Qiagen) was used according to manufacturer instructions. The product was purified with the QIAquick PCR Purification Kit (Qiagen) as per manufacturer's instructions and cloned using the TOPO TA system (Invitrogen). Recombinants were selected and inoculated into 96-well plates with Luria Burtani (LB) (Sambrook *et al.*, 1989) medium and 7.5% glycerol. Three 96-well plates were incubated overnight at 37°C and then stored at -80°C. Individual clones were grown in 5 ml LB overnight at 37°C and plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen) as per manufacturer's instructions. The sequence of each clone was determined using

the Big Dye Terminator chemistry with AmpliTaq-FS DNA polymerase (Applied Biosystems) and were performed at Cornell University BioResource Center using an Applied Biosystems Automated 3730 DNA Analyzer.

Phylogenetic Analysis

Sequences were analyzed using Sequencher DNA software (Gene Codes). Based on partial sequences, clones which were less than 95% similar to other clones from the library were selected for full-length sequence analyses. After removing chimeras via detection by Bellerophon version 3 (Huber *et al.*, 2004), sequences were loaded into the ARB program (Ludwig *et al.*, 2004), aligned, and phylogenetic trees were constructed. Operational taxonomic units (OTUs) in the community were determined by DOTUR (Schloss and Handelsman, 2005) analysis using ARB-constructed distance matrixes for the aligned 5´ and 3´ end of the rDNA gene. For these analyses, 99 full-length sequences and 129 partial sequences representing a mixture of the 5´ and 3´ end of the rDNA gene were used. DOTUR analysis was run on the distance matrixes from both the 3´ and 5´ end of the molecule. In order to have the most accurate reflection of saturation, we replaced the 3´ sequences with 5´ sequences that clustered with the same full length clone in an OTU at 97% similarity. We were then able to use DOTUR to evaluate all 228 sequences. The community composition was analyzed relative to other coral reef fish gastrointestinal microbial communities (Clements *et al.*, 2006) using Unifrac (Lozupone and Knight, 2005).

Investigation of the Archaeal Community

Archaeal genes were amplified from the *P. sexstriatus* hindgut community DNA using Archaeal-specific 16S rRNA gene primers ARC 856F and 1354R (Shinzato *et al.*, 1999), Met86F and 1340R (Wright and Pimm, 2003), and MLF and R, which targets the gene coding for the methylcoenzyme M reductase alpha-subunit (Hales *et al.*, 1996). Amplification conditions used were as described in Wright and Pimm (2003).

Clone library construction from the Met86F and 1340R reaction was conducted using the TOPO TA cloning system (Invitrogen) according to manufacturer's instructions. The library was screened for the presence of the insert using PCR and 21 clones were selected for sequence analysis (as described above). Additional sequences from close archaeal relatives were determined using the NCBI BLAST feature (http://www.ncbi.nlm.nih.gov/) and added into trees via ARB.

Fluorescent in situ *hybridization*

Fluorescent *in situ* hybridization was carried out as described by DeLong *et al.* (1989) with minor modifications. 10-well, multitest slides (ICN Biomedicals, Inc.) were prepared as in Ward and Angert (2008). Cell suspensions were washed twice with phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 1 L deionized H_2O, pH 8) and placed on wells. Cells were allowed to settle for 3 min, aspirated and air-dried. Cells were then treated with 1% paraformaldehyde (EM Grade, Electron Microscopy Sciences) for 20 min, room temperature, and washed twice with PBS. Freshly-made hybridization solutions (DeLong *et al.*, 1989) with 1.67 ng probe/µl and formamide concentrations of 10% for probe Eub 338, (Amann *et al.*, 1995), 30% for p687 (Icgen and Harrison, 2006), 20% for Arch 915, (Stahl and Amann, 1991), and 20% for Meth848 (AAGTCACGGCCTCTCTTCAA, this study) were added to wells and incubated in a humid hybridization chamber overnight at 37° C. Slides were washed three times in 0.2 x SET [diluted from 25x SET (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris (pH 7.8)] in a hybridization chamber at 37° C for a total of 3 washes. Wells with Eub338 or p687 (fluorescein-tagged) probes were then incubated for 10 min in 2.5 mg/ml Trypan Blue (Sigma) and washed twice in PBS to reduce autofluorescence. Then mounting media [10 µl of 1:1 Citifluor: PBS (Ted Pella, Inc.) with 0.02 mg/ml 4´, 6-diamidino-2-phenylindole (DAPI)] was added to all wells and the slides were covered, sealed and

viewed. Phase-contrast and fluorescence microscopy were performed using an Olympus BX61 epifluorescent microscope, with 40x UPlanFl (N.A. 0.75) and 100x UPlanApo 16 (N.A. 1.35) objectives. The microscope is equipped with filter cubes for viewing fluorescein, Cy3, and DAPI/Hoechst fluorescence. Images were acquired using a Cooke SensiCam and slidebook software (Intelligent Imaging Inc). Exposure times never exceeded 1.5 seconds. Figures were assembled using Adobe Illustrator CS. Quantification of the percentage of the community positive for hybridization was performed using the average from 10 randomly chosen fields of probe-positive cells/DAPI-positive cells.

Results:

Bacterial 16S rRNA Gene Clone Library Construction and Analysis:

After chimeras within the library were identified by Bellerophon (Huber *et al.*, 2004), a total of 228 clone sequences remained. Figure 1A shows the phylogenetic affiliation of the clones as analyzed by the myRDP version 9 Classifier program for phylum-level identification. Sequences affiliated with the Deltaproteobacteria (29.6%), *Bacteroidetes* (29.2%), and *Firmicutes* (19.8%) dominated the library. *Verrucomicrobia* (6.3%), *Lentisphaerae* (5.9%), *Deferribacteres* (3.4%), *Planctomycetes* (2.5%), Gammaproteobacteria (2.5%), *Spirochaeta* (0.4%), and Alphaproteobacteria (0.4%) made up the remaining phyla from the clone library. DOTUR analysis was carried out to assess the diversity of the community and the level of representation reflected in the clone library. Only clones more than 5% different from other clones in the library were sequenced entirely. Therefore our final library contained 99 full-length (1,484 bp) sequences and 129 partial sequences (≥500 bp) that were a mixture of the 5´ (75 sequences) and 3´ (54 sequences) end of the rDNA molecule. Because any subset of the library would be an underestimation of coverage, we carried out DOTUR analysis on both the 3´ and the 5´ end of the

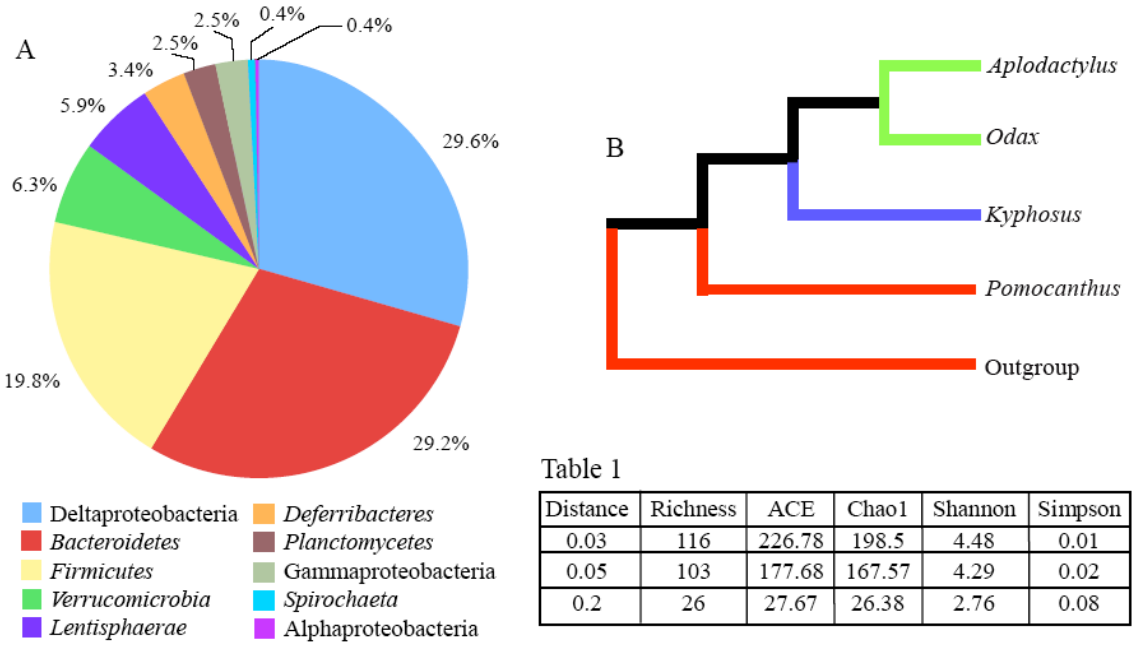


Table 1

Distance	Richness	ACE	Chao1	Shannon	Simpson
0.03	116	226.78	198.5	4.48	0.01
0.05	103	177.68	167.57	4.29	0.02
0.2	26	27.67	26.38	2.76	0.08

Figure 1. Phylum-level identification of the 228 sequences from the hindgut of *Pomacanthus sexstriatus* (A). The clone library was dominated by sequences affiliated with the Deltaproteobacteria, *Bacteroidetes*, and *Firmicutes.* A Unifrac Jackknife Environment Clustering with 100 repetitions found that the sequences from *Pomacanthus sexstriatus* were significantly different from *K. sydneyanus*, *O. pullus* and *A. arctidens*. Red lines indicate >99% of the trees supported the indicated branching (blue = >50%, green = < 50%). Measures of observed and estimated richness and diversity for all the sequences obtained from *Pomacanthus sexstriatus* via DOTUR analysis are shown in Table 1.

molecule cloned (Figure 2A). We then substituted 5´ sequences that clustered in the same operational taxonomic unit (OTU, distance 0.03) for 3´ sequences from that same OTU and carried out DOTUR on all 228 sequences (Table 1). Both the forward sequences and the combined forward and reverse sequences showed 116 OTUs at the "species" distance level of 0.03 (Table 1, Figure 2). The rarefaction curves for all three data sets failed to level off at the 0.03 distance (Figure 2 B). The abundance-based coverage estimator (ACE) and Chao1 estimator of richness of the environment predicted that our sample represents less than half of the species and genera (typically considered distance 0.5) that were present in this environment. At a distance of 0.03, Shannon values of >4 indicate large unevenness in distribution of OTUs in our sample. This unevenness diminished but did not disappear (complete evenness gives a Shannon index of 1) at the distance level 0.2, or 80% similarity. The Simpson numbers for our sample confirm high diversity in our samples.

Unifrac analysis was performed on microbial community sequences from three marine herbivorous fish *Kyphosus sydneyanus, Odax pullus* and *Aplodactylus arctidens* in comparison to the sequences obtained from *P. sexstriatus* (Clements *et al.*, 2006; Lozupone and Knight, 2005; Moran *et al.*, 2005). Jackknife community clustering was performed in order to detect differences in community clustering (Figure 1 B). Out of 100 replicates, the *Pomacanthus* community clustered separately from the other marine herbivorous fish communities greater than 99.9% of the time. Principle Component Analysis was performed on the samples from the four marine herbivorous fish (Figure 2 C). All comparisons showed the *Pomacanthus* community clustering in a different quadrant from the other fish microbial communities. A lineage-specific analysis was conducted to identify the clones that account for the greatest variability in community (Figure 3). The branch that separated Clostridial

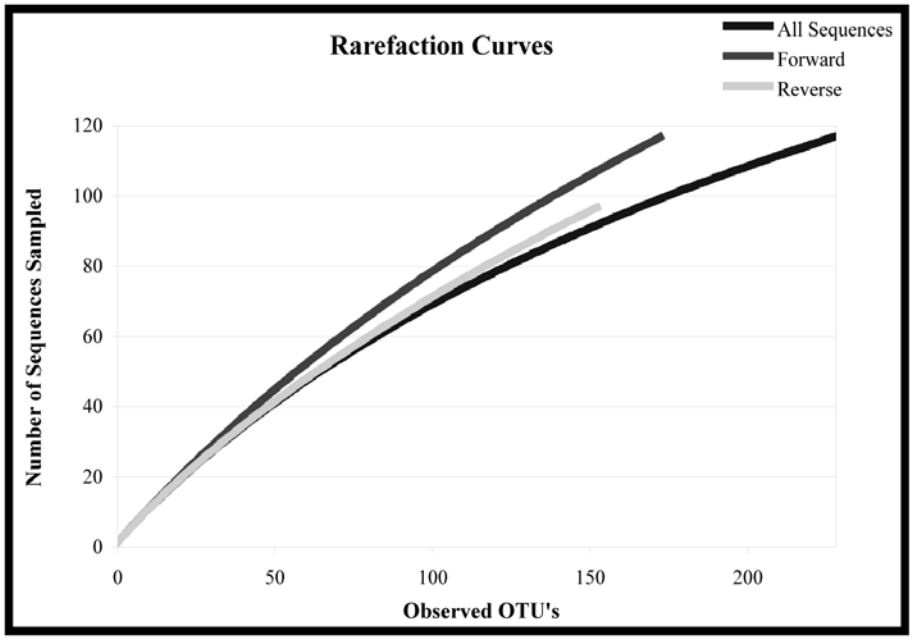
Figure 2.

DOTUR analysis was performed on the clone library sequences from *P. sexstriatus.* Clones that contained the eubacterial primer 8F are listed as Forward and those that contained 1492R are listed as Reverse. The DOTUR richness estimators predict that our sample represents less than half of the species (distance 0.03) and genera (distance 0.05) that were present in this environment (A). The rarefaction curves comparing Observed OTU's at a distance level of 0.03 (97% similarity) to the number of sequences sampled failed to level out for any of the three data sets, indicating the diversity of the sample was not exhausted (B). Unifrac Principle Component Analysis indicated that the *Pomacanthus* community (▼) clustered in a separate quadrant from the other marine herbivorous fish samples, *K. sydneyanus* (●), *O. pullus* (■) and *A. arctidens* (▲). The out-group used for this analysis was *M smithii* (▷).

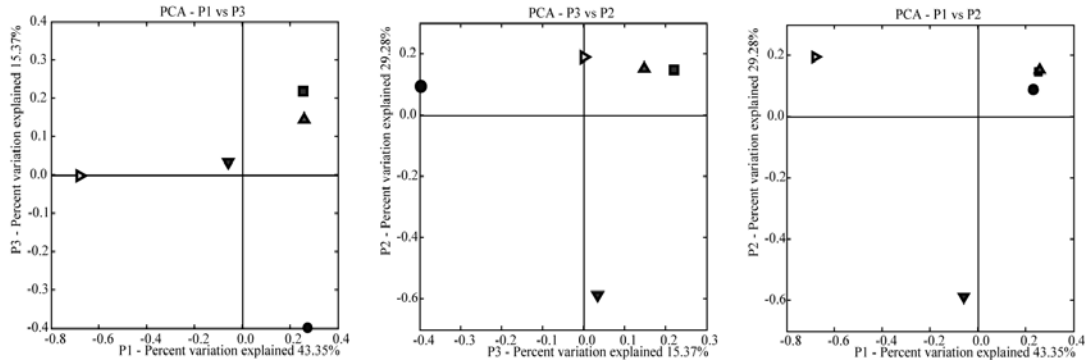
A

	distance	richness	ACE	Chao1	Shannon	Simpson
Forward Sequences Only	0.03	116	266.8	263.27	4.61	0.01
	0.05	104	237.38	207.55	4.44	0.01
	0.2	37	41.48	39.33	3.32	0.04
Reverse Sequences Only	0.03	96	283.23	215.9	4.34	0.01
	0.05	78	152.94	132.47	4.07	0.02
	0.2	22	26.94	25	2.45	0.12
Forward and Reverse Sequences	0.03	116	226.78	198.5	4.48	0.01
	0.05	103	177.68	167.57	4.29	0.02
	0.2	26	27.67	26.38	2.76	0.08

B



C

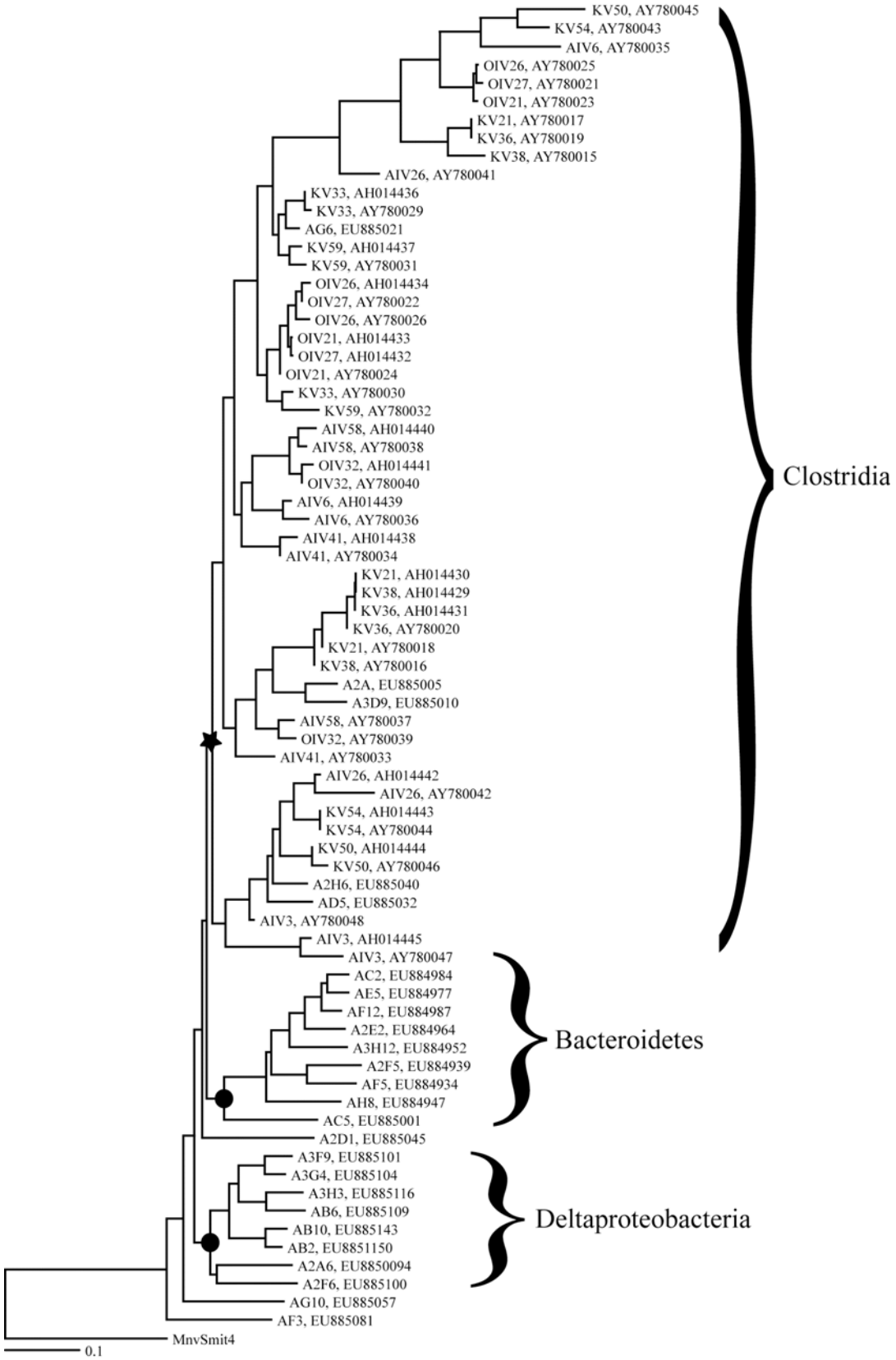


lineages from all other lineages was identified as a highly significant ($P=1.86e^{-09}$) while the branches that separated the *Bacteroidetes* and Deltaproteobacteria were considered marginally significant ($P=3.10e^{-02}$) and suggestive ($P=7.86e^{-02}$) respectively (branch length threshold 0.274391).

The phylogenetic trees constructed in ARB of the 100 sequences that were ≥5% different from other sequences in the library confirmed the trends seen using DOTUR (Fig 4). Nodes with a black dot indicate a bootstrap value above 70%. Several sequences associated with the Gammaproteobacteria or Alphaprotebacteria were represented, however they appeared to be a marginal component of the community (3%). Twenty-three of the sequences affiliated with the *Bacteroidetes*, a common component of gastrointestinal systems (Choat and Clements, 1998; Karasov and Martinez Del Rio, 2007). *Lentasphaerae*-associated sequences were not particularly abundant, but for their small representation, they appear diverse. The sequences that clustered with the Verrucomicrobia were related to organisms found in with *Planctomycetes* and *Deferribacterales* were anaerobic environments such as cow rumens and landfills. Sequences affiliated with the *Planctomycetes* and *Deferribacterales* were also minor components of the community. The insets showing the sequences associated with the Deltaproteobacteria and *Firmicutes* contained 24 clones and 27 clones, respectively (Fig 5 A and B). The *Firmicutes*-associated sequences appear quite phylogenetically diverse, containing 27/99 of the operational taxonomic units (OTU, distance 0.05) while only representing 20% of the overall sequences (see Figure 1). The majority of the sequences clustering with the Deltaproteobacteria affiliate with the order *Desulfovibrionales* although some of the sequences are associated with other known sulfur-reducing lineages.

Figure 3.

This Unifrac lineage-specific analysis shows all the sequences used for community comparison from *K. sydneyanus, O. pullus* and *A. arctidens* as well as the *P. sexstriatus* clones from this study. This analysis identified the clones that accounted for the greatest variability in community. The branch that separated Clostridial lineages from all other lineages (black star) was identified as a highly significant (P=1.86e-09) while the branches that separated the *Bacteroidetes* and Deltaproteobacteria (black circles) were considered marginally significant (P=3.10e-02) and suggestive (P=7.86e-02) respectively (branch length threshold 0.274391). Accession numbers follow the first letter of the fish genus name, intestinal segment number, and fish collection number for the Clements *et al.* samples. *Pomacanthus sexstriatus* samples are labeled with then clone name followed by the accession number.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KV36, AY780019
KV38, AY780015
AIV26, AY780041
KV33, AH014436
KV33, AY780029
AG6, EU885021
KV59, AH014437
KV59, AY780031
OIV26, AH014434
OIV27, AY780022
OIV26, AY780026
OIV21, AH014433
OIV27, AH014432
OIV21, AY780024
KV33, AY780030
KV59, AY780032
AIV58, AH014440
AIV58, AY780038
OIV32, AH014441
OIV32, AY780040
AIV6, AH014439
AIV6, AY780036
AIV41, AH014438
AIV41, AY780034
KV21, AH014430
KV38, AH014429
KV36, AH014431
KV36, AY780020
KV21, AY780018
KV38, AY780016
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A3D9, EU885010
AIV58, AY780037
OIV32, AY780039
AIV41, AY780033
AIV26, AH014442
AIV26, AY780042
KV54, AH014443
KV54, AY780044
KV50, AH014444
KV50, AY780046
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AD5, EU885032
AIV3, AY780048
AIV3, AH014445
AIV3, AY780047
AC2, EU884984
AE5, EU884977
AF12, EU884987
A2E2, EU884964
A3H12, EU884952
A2F5, EU884939
AF5, EU884934
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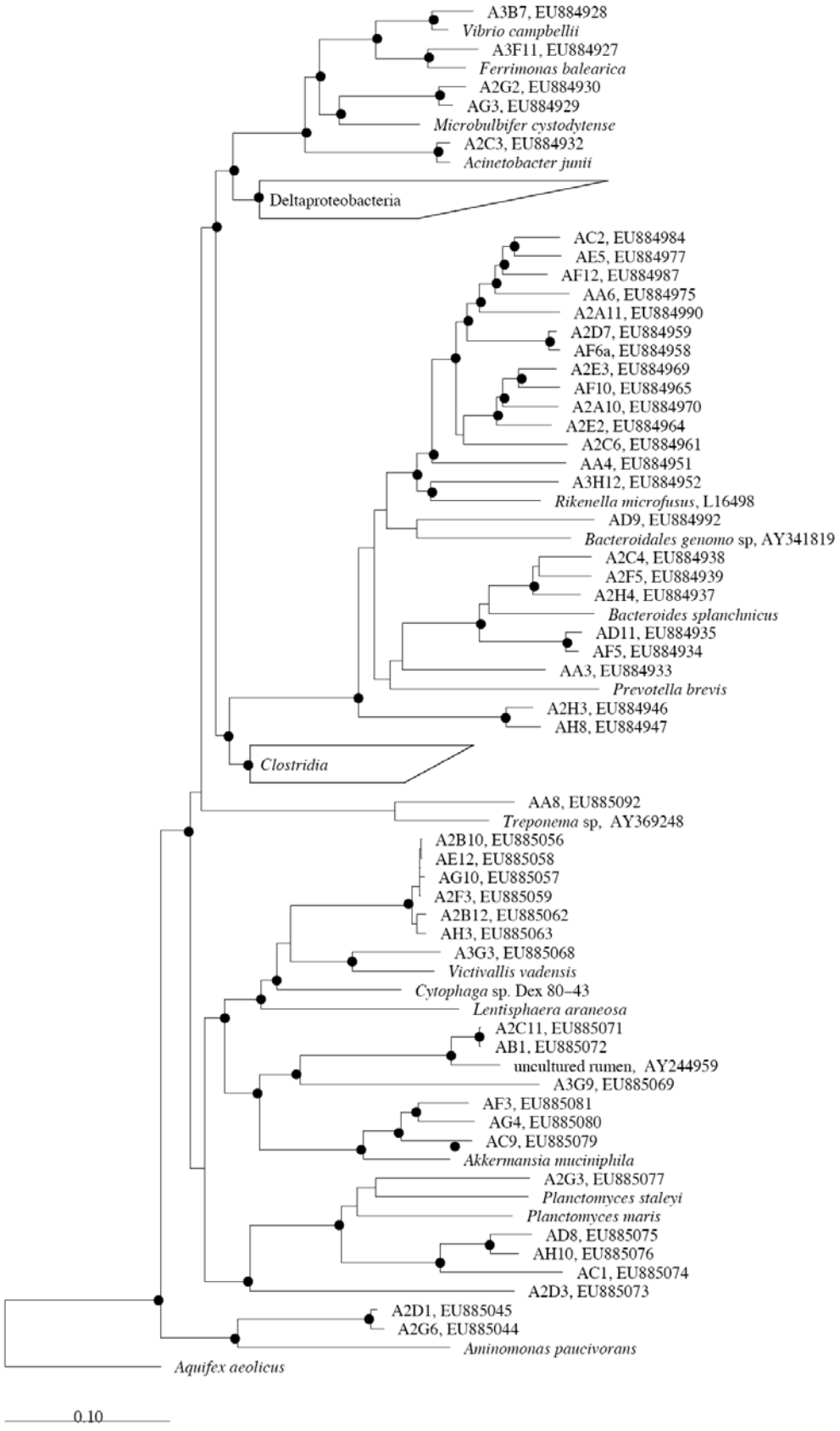
A3F9, EU885101
A3G4, EU885104
A3H3, EU885116
AB6, EU885109
AB10, EU885143
AB2, EU8851150
A2A6, EU8850094
A2F6, EU885100
AG10, EU885057
AF3, EU885081
MnvSmit4
0.1
Clostridia
Bacteroidetes
Deltaproteobacteria

Figure 4.

Phylogenetic analysis of the sequences obtained from the *P. sexstriatus* hindgut community. Sequences represent clones that were >95% different from other clones obtained from this sample and were therefore sequenced from position 8-1492 (*E.coli* numbering). Accession numbers are listed next to all uncultured organisms. Neighbor-joining bootstrap values above 70% are indicated (black circles).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Ferrimonas balearica
A2G2, EU884930
AG3, EU884929
Microbulbifer cystodytense
A2C3, EU884932
Acinetobacter junii
Deltaproteobacteria
AC2, EU884984
AE5, EU884977
AF12, EU884987
AA6, EU884975
A2A11, EU884990
A2D7, EU884959
AF6a, EU884958
A2E3, EU884969
AF10, EU884965
A2A10, EU884970
A2E2, EU884964
A2C6, EU884961
AA4, EU884951
A3H12, EU884952
Rikenella microfusus, L16498
AD9, EU884992
Bacteroidales genomo sp, AY341819
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A2F5, EU884939
A2H4, EU884937
Bacteroides splanchnicus
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AF5, EU884934
AA3, EU884933
Prevotella brevis
A2H3, EU884946
AH8, EU884947
Clostridia
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Treponema sp, AY369248
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AE12, EU885058
AG10, EU885057
A2F3, EU885059
A2B12, EU885062
AH3, EU885063
A3G3, EU885068
Victivallis vadensis
Cytophaga sp. Dex 80–43
Lentisphaera araneosa
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AB1, EU885072
uncultured rumen, AY244959
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AF3, EU885081
AG4, EU885080
AC9, EU885079
Akkermansia muciniphila
A2G3, EU885077
Planctomyces staleyi
Planctomyces maris
AD8, EU885075
AH10, EU885076
AC1, EU885074
A2D3, EU885073
A2D1, EU885045
A2G6, EU885044
Aminomonas paucivorans
Aquifex aeolicus
0.10

Archaeal Community Analysis

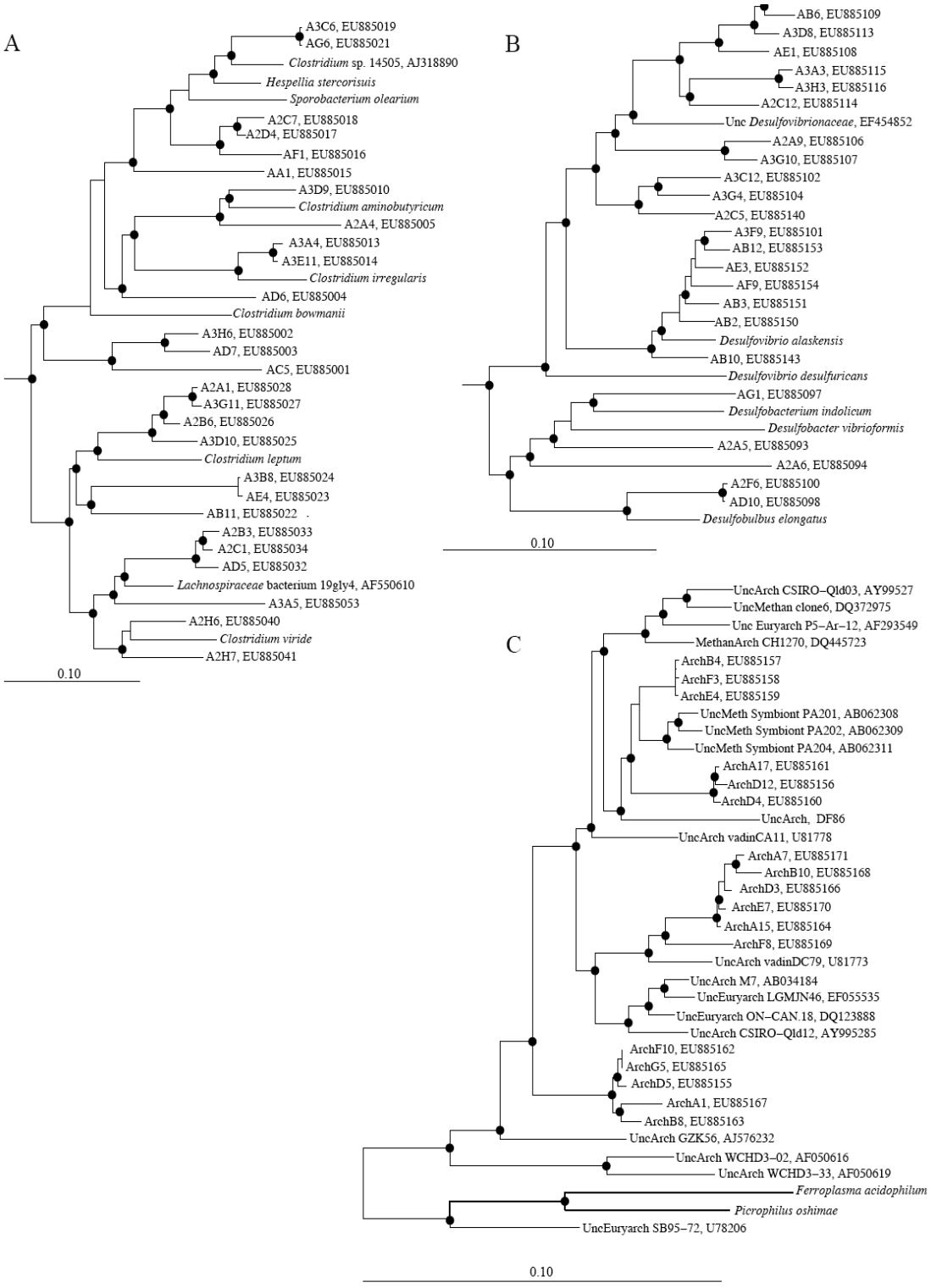
The presence of organisms carrying 16S rRNA gene sequences associated with the domain Archaea, specifically the methanogens, and a methanogen-associated functional gene (McrA) was confirmed via PCR (data not shown). The Archaeal 16S rRNA gene sequences obtained from the hindgut community DNA are shown in Figure 5C. These sequences were closely related to each other and only distantly related to a cultured relative *Picrophilus oshimae*, all within the *Thermoplasmatales.* In order to construct an appropriate phylogenetic tree for these sequences, sequences from uncultured relatives were found via NCBI BLAST search.. The related sequences were derived from clone libraries from the microbial communities found in the rumen of cows and sheep (Wright *et al.*, 2006; Wright *et al.*, 2007), the gut of termites (Shinzato *et al.*, 2001) and xylophagous cockroaches (Hara *et al.*, 2002; Shinzato *et al.*, 1999; Shinzato *et al.*, 2001), and anaerobic digesters (Boonapatcharoen *et al.*, 2007; Huang *et al.*, 2003).

Fluorescent in situ *Hybridization*

Confirmation of presence and relative abundance of microorganisms related to the *Desulfovibrio* and the *Thermoplasmales*-associated Archaea was carried out using fluorescent *in situ* hybridization. Micrographs of representative fields depicting the hybridization of the Meth848 probe were taken (Fig 6 A, Meth848 in red overlaid with the blue DAPI-stained cells). The morphology of this group appeared quite diverse and the cells were relatively abundant. Direct counts of 10 randomly chosen fields found the Meth848 probe hybridized to 4 % of the DAPI-stained cells. The *Desulfovibrio*-specific p687 probe (Fig 6 C, in green) also hybridized to cells with diverse morphology, including but not limited to the characteristic vibroid or spirillum morphology.

Figure 5.

Insets from the phylogenetic tree in Figure 1 (A, B). Twenty-seven clones associated with *Clostridiales* were sequenced in full. These sequences cluster with diverse Clostridial lineages (A). The Deltaproteobacteria dominated the clone library from *P. sexstriatus*. Most of these sequences were associated with the *Desulfovibrionacae* (B). Archaeal clones obtained from the hindgut of *P. sexstriatus* (C). All of the clones affiliate with sequences from putative methanogens from environments such as sheep and cow rumens, termite hindguts, and anaerobic digesters (C). This group of Archaea is distantly related to members of the *Thermoplasmatales*. Neighbor-joining bootstrap values above 70% are indicated (black circles).


A
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Clostridium sp. 14505, AJ318890
Hespellia stercorisuis
Sporobacterium olearium
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A2D4, EU885017
AF1, EU885016
AA1, EU885015
A3D9, EU885010
Clostridium aminobutyricum
A2A4, EU885005
A3A4, EU885013
A3E11, EU885014
Clostridium irregularis
AD6, EU885004
Clostridium bowmanii
A3H6, EU885002
AD7, EU885003
AC5, EU885001
A2A1, EU885028
A3G11, EU885027
A2B6, EU885026
A3D10, EU885025
Clostridium leptum
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AE4, EU885023
AB11, EU885022
A2B3, EU885033
A2C1, EU885034
AD5, EU885032
Lachnospiraceae bacterium 19gly4, AF550610
A3A5, EU885053
A2H6, EU885040
Clostridium viride
A2H7, EU885041
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B
AB6, EU885109
A3D8, EU885113
AE1, EU885108
A3A3, EU885115
A3H3, EU885116
A2C12, EU885114
Unc Desulfovibrionaceae, EF454852
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A3G10, EU885107
A3C12, EU885102
A3G4, EU885104
A2C5, EU885140
A3F9, EU885101
AB12, EU885153
AE3, EU885152
AF9, EU885154
AB3, EU885151
AB2, EU885150
Desulfovibrio alaskensis
AB10, EU885143
Desulfovibrio desulfuricans
AG1, EU885097
Desulfobacterium indolicum
Desulfobacter vibrioformis
A2A5, EU885093
A2A6, EU885094
A2F6, EU885100
AD10, EU885098
Desulfobulbus elongatus
0.10
C
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UncMethan clone6, DQ372975
Unc Euryarch P5–Ar–12, AF293549
MethanArch CH1270, DQ445723
ArchB4, EU885157
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ArchA15, EU885164
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ArchA1, EU885167
ArchB8, EU885163
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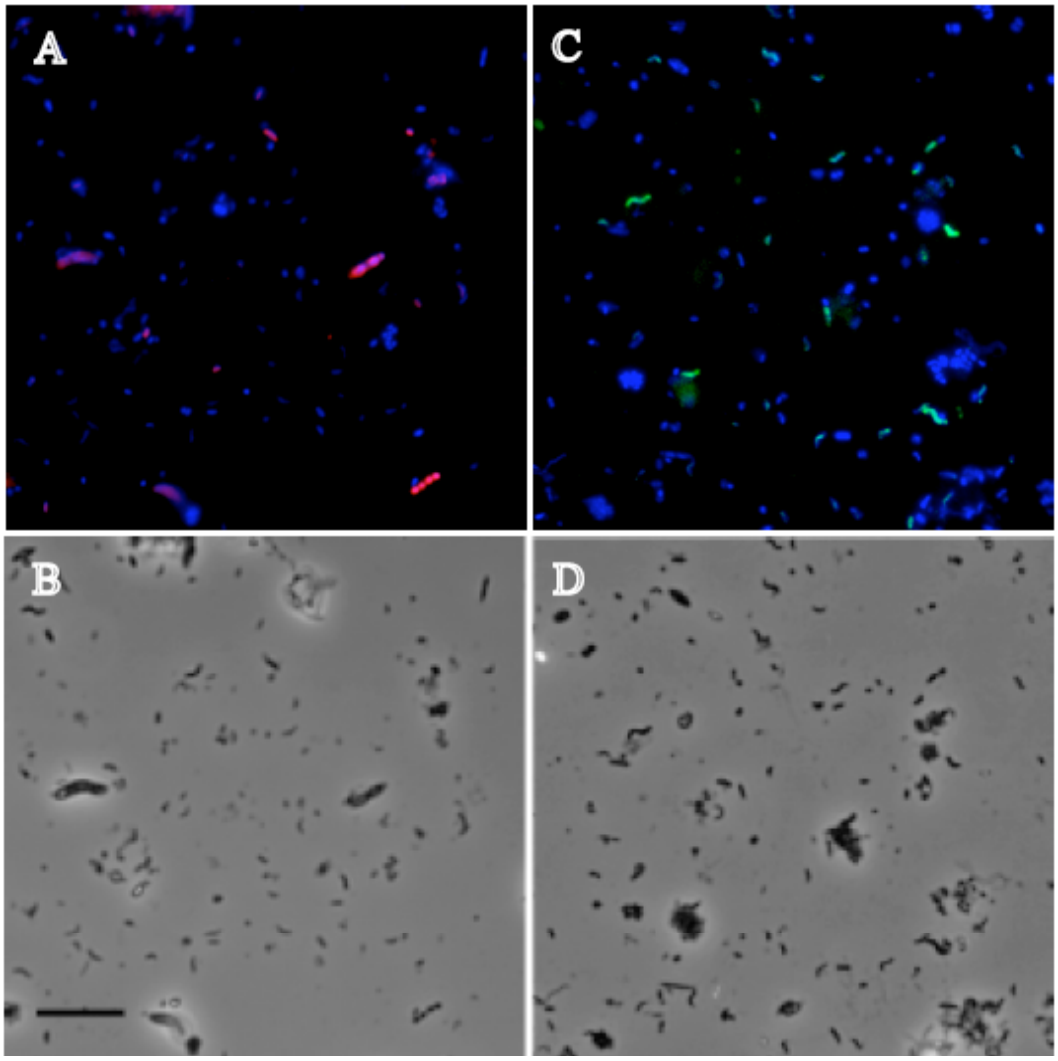

Figure 6. Fluorescent *in situ* hybridization (FISH) was carried out on samples from the hindgut of *P. sexstriatus*. The presence of Archaea distantly related to the *Thermoplasmatales* was confirmed (A, B). The Archaeal probe is shown in red while DAPI staining is shown in blue. *Desulfovibrio*-associated cells are shown in green (C).

Direct counts indicated that p687 hybridized to 34% of the DAPI-stained cells. The Eub338 and Arch915 probes acted as positive controls (data not shown).

Discussion:

Although several authors have made important contributions recently, there is a dearth of studies on the gastrointestinal microbial community of marine fish. Thus we are only beginning to grasp the factors that shape these communities. However, enough data has been gathered to begin to make hypothesis-generating comparisons. In this study we survey the microbial community of a single angelfish of the species *P. sexstriatus*. We used two computer-based tools, DOTUR and Unifrac, to quantify diversity, richness, and other ecological indicators within our clone library (Schloss and Handelsman, 2005) and compare our community to consortia from other environments (Lozupone and Knight, 2005). We also conducted a qualitative analysis of the community through a description of the phylogenetic affiliations of the clones. Finally, we confirmed lineage representation in this community using fluorescent *in situ* hybridization.

DOTUR analysis of our clone library indicates that we had not uncovered the full extent of that diversity. The rarefaction curves reveal that additional OTUs at a 97% similarity cut-off would have been uncovered if more clones were obtained from the sample. The DOTUR richness estimators predict that our sample represents less than half of the genera that were present in this environment. We also had a highly uneven distribution of sequences falling into our 116 OTUs (97% similarity) as indicated by the high Shannon index. Some "species" were represented considerably more heavily than others. The measure of the diversity of the population, the Simpson index, calculates the probability that two individuals randomly selected from a sample will belong to the same OTU at a certain distance. It takes into account the number of organisms present, as well as the abundance of each organism. The Simpson numbers

for our sample confirm high diversity in this community. All these analyses of the clone library reveal that this is a microbial population with a variety of different organisms that range widely in representation.

Community analysis using Unifrac revealed significant differences between the *Pomacanthus* microbiota and that of marine herbivorous fish (Clements *et al.*, 2006; Lozupone and Knight, 2005; Moran *et al.*, 2005). In Clements *et al.* (2006) sequences from *K. sydneyanus*, *O. pullus* and *A. arctidens* were analyzed by Amplified Ribosomal DNA Restriction Analysis (ARDRA) and OTUs were identified based on a cutoff of 4 or more clones with that restriction pattern. In order to compare analogous samples, we used DOTUR to identify OTUs with 3 or more representatives and compared them with the previously published studies using Unifrac. Jackknife Environment Clustering and PCA analysis of the samples both indicate major microbial composition differences between *P. sexstriatus* and *K. sydneyanus*, *O. pullus* and *A. arctidens* (Figure 1 B, and 2 C). A lineage-specific analysis to identify the clones that account for the greatest variability in community structure identified the branch that separated the Clostridial lineages from all other lineages as a highly significant difference (Figure 3). The Deltaproteobacteria, *Bacteroidetes*, and other lineages found in the hindgut of *P. sexstriatus* make this community significantly different from other marine herbivorous fish microbial communities.

Although *Desulfovibrio*-associated sequences have been obtained from the intestinal tracts of the marine herbivorous fish *Hermosilla azurea* (Fidopiastis *et al.*, 2006), they were a minor component of a biota dominated by organisms with *Enterovibrio*-afilliated sequences. It has been suggested that the protocol used to obtain DNA for this analysis may have favored selective lysis of Proteobacteria over Firmicutes, especially given the remarkable lack of *Clostridia*-associated sequences found in the study (Clements *et al.*, 2006). Gram-positive bacterial lineages routinely

dominate gastrointestinal ecosystems (Moran *et al.*, 2005; Nelson *et al.*, 2003). Given the diversity of the sequences found in this study that clustered with the *Clostridiales* and our failure to exhaust the diversity of the sample, we conclude that Clostridial lineages are a major component of the community in this sample as well. The direct DNA extraction method and few rounds of PCR amplification used herein were suitable for representation of microbial diversity. This supports the conclusion that the unusual prevalence of Gram-negative *Desulfovibrio*-like sequences in this study was an accurate reflection of the environment. The fluorescent *in situ* hybridization direct counts of *Desulfovibrio*-like cells provides confirmation that organisms closely related to known sulfur reducing bacteria (SRB) are a large component of the bacterial population in this sample.

An additional remarkable feature of this particular environment was the presence of organisms closely related to an unusual group of putative methanogens. The Archaea found in this environment are distantly related to *Picrophilus oshimae* of the *Thermoplasmales.* They are much more closely related to sequences from rumens, insect gastrointestinal tracts, and anaerobic digesters (Boonapatcharoen *et al.*, 2007; Hara *et al.*, 2002; Huang *et al.*, 2003; Shinzato *et al.*, 1999; Shinzato *et al.*, 2001; Wright *et al.*, 2006; Wright *et al.*, 2007). This phylogenetic affiliation, confirmation of presence of these cells via fluorescent *in situ* hybridization and successful amplification of the functional gene McrA suggests these organisms may carry out methanogenesis in the gastrointestinal tract of *P. sexstriatus.*

The co-incidence of both a large population of bacteria capable of sulfate reduction and a significant group of Archaea capable of methanogenesis is also of interest. The coexistence of these types of organisms has been subjected to kinetic analysis that found that SRB's and methanogens compete for hydrogen and acetate in freshwater lake sediments (Lovely *et al.*, 1982). SRBs use the acetate as a carbon source and

hydrogen is used for the reduction of sulfate into H_2S. Methanogenic substrates are restricted to three major types: CO_2, methyl-group containing compounds, and acetate (Liu and Whitman, 2008). The factors which appear to modulate the prevalence and activity of the two populations include sulfate availability, the partial pressure of hydrogen, and, for acetoclastic methanogens, the acetate levels (Lovely *et al.*, 1982; Stams *et al.*, 2005; Stolyar *et al.*, 2007). However, these groups of organisms routinely co-exist in gastrointestinal systems and no negative correlation between the two population sizes was detected in a recent study in humans (Stewart *et al.*, 2006). Investigations into the ecological dynamics of anoxic marine sediments revealed a thriving community of both SRBs and methanogens which utilized the algal osmoprotectant dimethylsulfoniopropionate (DMSP) (Kiene and Visscher, 1987). But measurements conducted in other marine herbivorous fish of sulfate reduction and methanogenesis using radio-labeled acetate have revealed that these processes played only a minor role in overall anaerobic metabolic function (Mountfort *et al.*, 2002). Methanogenesis accounted for only ~1% of acetate turnover, while sulfate reduction was responsible for less than 5% of the CO_2 produced. The authors speculate that short retention time of substrates within the gastrointestinal tract combined with long doubling times of SRBs were factors driving the low sulfate reduction rates (Mountfort *et al.*, 2002). However, none of these fish species appear to harbor large SRB populations (Clements *et al.*, 2006).

Although the substrates to sustain such a large population of SRBs in *P. sexstriatus* have yet to be identified, an intriguing hypothesis may be drawn from these data. Refractory sulfonated carbon compounds are a common algal defense mechanism against predation (Percival and McDowell, 1967). DMSP is also widespread within a variety of algae, including those found in the habitat of *P. sexstriatus* (Broadbent *et al.*, 2002; Van Alstyne, 2008). If the microbiota found in this study were involved in

anaerobic metabolism of these sulfur-containing carbon compounds, this could significantly expand the dietary range of these angelfish (Martinez-Diaz and Perez-Espana, 1999; Zemke-White and Clements, 1999). This in turn could have larger ecological implications, given recent findings on the importance of herbivory in coral reef resilience (Hughes *et al.*, 2003; Hughes *et al.*, 2007; Mumby *et al.*, 2006; Mumby *et al.*, 2007). Clearly, the physiology of this community remains to be explored and additional fish should be examined. However, our findings on the prevalence of SRBs combined with the presence of an unusual group of putative methanogens in *P. sexstriatus* widen our understanding of these microbial communities, so central for fish digestion, and thus for coral reef ecology as a whole.

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APPENDIX A

CYTOSKELETAL ELEMENTS IN *EPULOPISCIUM* SP TYPE B

Introduction

Recent advances in bacterial cell biology have demonstrated the importance of the bacterial cytoskeleton for processes as diverse as proper insertion of membrane proteins to chromosome segregation. *Epulopiscium* sp. Type B is an unusually large bacterium (~250 μm in length) that reproduces solely by the formation of multiple internal offspring. These cells contain a substantial amount of DNA throughout their developmental cycle and this DNA is composed of tens to hundreds of thousands of copies of its genome. However, only a small portion of the total DNA is packaged into offspring. In order to further understand the mechanisms regulating cell size and chromosome segregation in *Epulopiscium* we studied the actin-like *mreB* gene in this organism. We cloned *Epulopiscium mreB,* expressed the protein, and produced antiserum capable of detecting MreB in lysate from surgeonfish gut contents that contain *Epulopiscium*. Preliminary results indicate this protein may form sub-cellular structures near the poles.

Background

The importance of the eukaryotic cytoskeleton has long been established but recent advances in microscopy and genomics have led to the elucidation of similar and related structures among bacteria as well (Graumann, 2007; Pogliano, 2008). The best-characterized components of bacterial cytoskeleton are the proteins FtsZ and MreB. These proteins are homologues of the eukaryotic tubulin and actin proteins, respectively. Ongoing research will reveal the extent of their functional homology to their eukaryotic counterparts, but their structural homology is confirmed (Graumann, 2007). Cell shape and width regulation and cell wall synthesis/maintenance have been

indicated as functions of MreB (Carballido-Lopez and Formstone, 2007; Divakaruni *et al.*, 2007). Additionally, there is some evidence that MreB may play a role in bacterial differentiation in some species (Mazza *et al.*, 2006) and chromosome segregation in others (Gitai *et al.*, 2005). In this study, we hoped to explore MreB regulation and localization in *Epulopiscium* sp. Type B. Investigations of the role of the cytoskeletal component MreB in *Epulopiscium* may provide insights into both the maintenance of large cell size and the unusual DNA dynamics in this organism.

Materials and Methods

The *Epulopiscium mreB* gene was identified from preliminary genome data via BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Because many bacteria have more than one homologue of MreB, we compared protein sequences from a variety of Low G+C Gram positive bacteria using Clustal X, PHYLIP, and TreeView to verify that we had cloned the appropriate gene (data not shown). A portion of the *Epulopiscium mreB* gene (735 of a total 1025 predicted base pairs, positions 139-874) was amplified from genomic DNA with the primers MrebF and MreBR (GCAGTTGGAGATGAAGCAAAA, TCCTGTTAAAACGATTCC). The fragment was cloned into a TOPO TA vector according to manufacturer instructions (Invitrogen). Using primers MreBF1 and MreBR1 including 5' recognition sites for the restriction enzymes *Nco*I and *Bam*HI respectively, the fragment was amplified from the TOPO TA vector, purified (Qiagen PCR Purification Kit), and cloned into the expression vector pET 28a according to manufacturer instructions and transformed into *E. coli* DH5α competent cells (Novagen). Expression of the MreB fragment from this vector would produce a His-tagged peptide. After verification using PCR amplification of the MreB fragment, the pET28a:*mreB* vector was transformed into *E. coli* BL21 cells and the MreB fragment was expressed through IPTG induction as per manufacturer instructions.

Protein purification was carried out using a Ni-NTA agarose resin (Qiagen). Western blot analysis (Sambrook *et al.,* 1989) showed bands of appropriate size reacting with both anti-His and *B. subtilis* anti-MreB antisera (courtesy of J. Errington) from the total lysate and the His-tag purified protein fraction. Following Bradford assay quantification of the *Epulopiscium* MreB fragment (Sambrook *et al.,* 1989), total purified protein was used for rabitt antibody production (Convance). The final antibody specificity was verified via Western blot using the His-tagged MreB fragment. Rabbit anti-MreB and anti-rabbit conjugated to alkaline phosphatase (Promega) was then used to evaluate *Epulopiscium* MreB expression in samples taken from two different samples of surgeonfish gut contents using Western blots. Immunodetection of MreB was carried out in wells of approximately 2 x 1 cm hand-drawn onto microscope slides (Corning, Corning, NY) using a hydrophobic PAP Pen (Daido Sangyo Co., Tokyo, Japan). These wells were treated with 0.1% poly-L-lysine, for 3 min, then the solution was removed by aspiration, and the wells rinsed with deionized water. A sample of *Epulopiscium* cells was washed three times with GTE (50 mM glucose, 25 mM Tris-Cl, pH 8, 10 mM EDTA). Each time, cells were pelleted by centrifugation at 3,000 G (Eppendorf Micro Centrifuge 5451 C, Westbury, NY) to remove smaller debris. Cells were resuspended in GTE, placed in the poly-lysine coated wells and incubated 4 min at room temp. Wells were aspirated and allowed to air dry completely. Cells were permeabilized by incubation in a lysis solution (5 mg/ml lysozyme, 2 mg/ml achromopeptidase, 2,000 U/ml mutanolysin in GTE) for one hour at 37°C in a humid chamber. Anti-MreB was added to the wells at 1:500 in 2 X DIG High Prime DNA Labeling and Detection Kit Blocking Reagent and incubated overnight at 4° C. The wells were washed with PBS ten times and a secondary antibody solution [Cy3-anti-rabbit antibody (Molecular Probes) in 2 X DIG blocking buffer] was added to the wells and allowed to incubate for 1 hour at room

temperature. Wells were then washed ten times in PBD and coverslips were mounted in 1:1 Citifluor (Ted Pella, Inc., Redding, CA) plus deionized H_2O, and 0.02 mg/ml 4',6-diamidino-2-phenylindole (DAPI).

Phase-contrast, Nomarski DIC and fluorescence microscopy were performed using an Olympus BX61 epifluorescence microscope, with 40x UPlanFl (N.A. 0.75) and 100x UPlanApo (N.A. 1.35) objectives. The microscope is equipped with filter cubes for viewing fluorescein, Cy-3, and DAPI/Hoechst fluorescence. Images were acquired using a Cooke SensiCam (Cooke Corp., Romulus, MI) and Slidebook software (Intelligent Imaging Inc., Denver, CO). Figures were assembled using Adobe Illustrator CS.

Results

Gene arrangement and sequence similarity suggested that *Epulopiscium* contained one MreB homologue (Figure 1). Protein expression was verified though SDS-PAGE

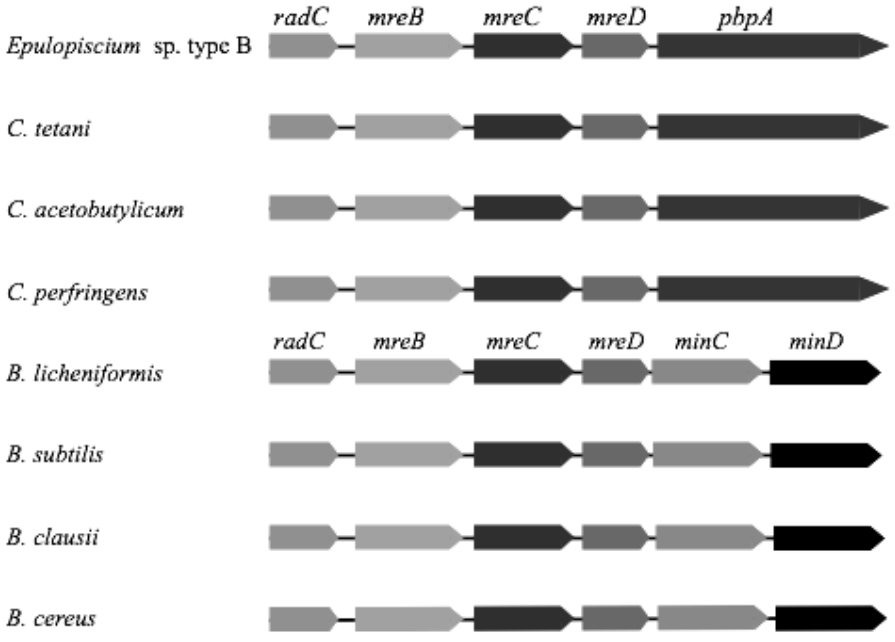

Figure 1. Sequence similarity and genomic context were examined in order to identify the appropriate MreB homologue in *Epulopiscium.*

analysis of total protein from *E. coli*, *B. subtilis*, *C. lentocellum* and His-tag purified protein from the *E. coli* with pET28a (Figure 2). After antibody production we confirmed that total lysate from *Epulopiscium* samples contained a protein that cross-reacted with the anti-MreB with Western Blot analysis (Figure 3). Preliminary results from immunodetection of MreB in whole *Epulopiscium* cells suggest clustering of signal around the poles of the mother cell and around the condensed DNA at the tips of the daughter cells (Figure 4).

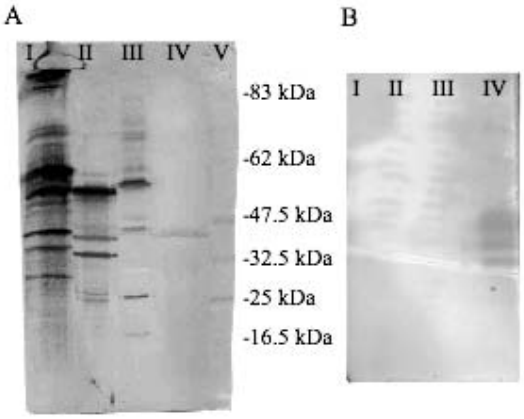

Figure 2. SDS PAGE analysis of total protein extracts (A) from *E. coli* (I), *B. subtilis* (II), *C. lentocellum* (III), and His-tag purified insert from pET28a (IV). Size marker is shown (V). Western blot analysis using *B. subtilis* anti-MreB (B) of *E. coli* DH5α (I), *E. coli* DH5α with pET28a without insert (II), *E. coli* DH5α with pET28a:*mreB* uninduced (III), and *E. coli* DH5α with pET28a:*mreB* after induction (IV). Lane IV appears to show some degradation products as well as a full size fragment of MreB.

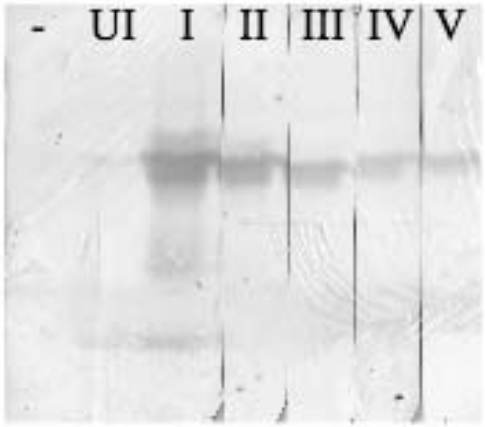

Figure 3**.** Western blot analysis of samples using the *Epulopiscium* anti-MreB. Lanes indicate *E. coli* DH5α (-), *E. coli* DH5α with pET28a:*mreB* uninduced (UI), and *E. coli* DH5α with pET28a:*mreB* after induction (I). Lanes II-III are 1:100 and 1:500 anti-MreB probing of total protein sample from a single *Naso tonganus*. Lanes IV and V are the same analysis of total protein from another *N. tonganus.*

Discussion

MreB plays a central role in regulating shape and size of a variety of bacteria (Carballido-Lopez and Formstone, 2007; Graumann, 2007; Pogliano, 2008). There is also data which suggests a role for MreB in chromosome segregation (Defeu Soufo and Graumann, 2005, 2006; Kruse *et al.*, 2006). *Epulopiscium* sp. type B MreB could aid the maintainence of an unusually large cell and potentially regulate the segregation or partitioning of the remarkable number of genomes (Mendell *et al.*, 2008). Investigation into *Epulopiscium* MreB could contribute to understanding the functional diversity of this important cytoskeletal element. In these studies, I confirmed that *Epulopiscium* contains a putative MreB homologue and produced anti-MreB capable of detecting the protein in total lysate of *Epulopiscium* samples. My preliminary work detecting MreB localization within whole cells suggest this protein may have distinct localization corresponding to the poles of mother cells and

offspring. This may indicate that *Epulopiscium* MreB plays a role in either shape determination or chromosome segregation into the developing offspring. However, further work must be done to support these initial findings. Some of the immunolocalization data demonstrated what appeared to be non-specific staining, suggesting that the detection protocol should be modified. Also, the antibody should be affinity purified to reduce non-specific attachment to sample debris. *Epulopiscium* samples from a variety of developmental stages should be examined to determine if localization changes with development. Finally, the use of confocal microscopy to visualize any three-dimensional structures formed by MreB would also advance our understanding of MreB dynamics during *Epulopiscum* development.

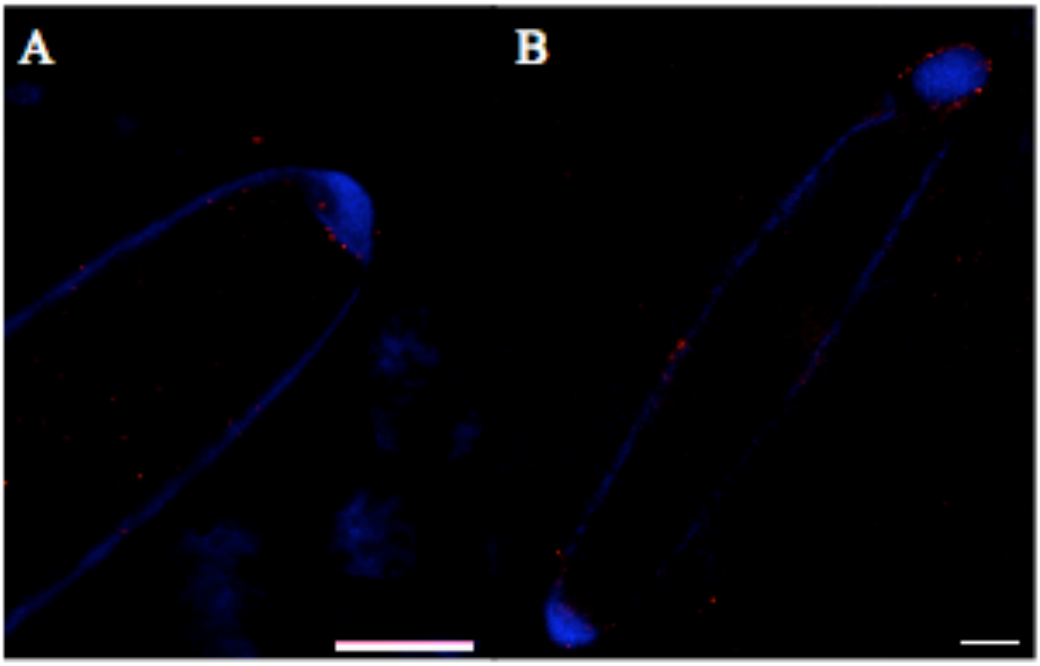

Figure 4. Preliminary data from *Epulopiscium* daughter cells suggests there may be clustering of MreB around the condensed DNA at the poles (A, B). In these micrographs, DNA is shown in blue and MreB localization is in red. Scale bar is

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APPENDIX B

CRYOFIXATION OF *EPULOPISCIUM* SP TYPE B

Introduction

New electron microscopy techniques have made enormous advances in high resolution imaging of subcellular structures in recent years. These data have been part of a body of work that has altered the conception of the bacterial cell, formerly viewed as only a "bag of enzymes." The large bacterium *Epulopiscium* sp. type B appears to have unusual cytoarchitecture which may be functionally important for maintenance of the ~250 micron long cigar-shaped cell. In this preliminary study, I utilized cryofixation, freeze substitution, and electron microscopy of Naso tonganus gut contents to examine the ultrastructure of *Epulopiscium* sp. type. B. Although interpretation of the results was complicated by poor sample quality, these studies may aid further efforts to investigate subcellular structures in this remarkable bacterium.

Background

Large *Epulopiscium* cells contain a tremendous amount of DNA composed of tens to hundreds of thousands of copies of the genome (Mendell *et al.*, 2008). These cells appear to localize this DNA in close proximity to the cell membrane as seen in initial high-resolution electron microscopy and fluorescence microscopy images of conventionally fixed cells (Robinow and Kellenberger, 1994; Robinow and Angert, 1998). This arrangement is in contrast to high-resolution images of nucleoid arrangement in most model organisms that depict a centrally located but highly dynamic cellular body (Dame, 2005; Lewis, 2004; Sherratt, 2003). Imaging of the subcellular components of the bacterial cell has made significant advances in recent years with the emergence of electron microscopy techniques that reveal near-native conformations of cellular constituents (Vanhecke *et al.*, 2008). These advances made

possible the recognition of tendrils of DNA that could accommodate transertion, or the linked process of transcription, translation and insertion of proteins into the membrane (Norris and Madsen, 1995; Norris *et al.*, 1996). They also began to reveal alternate nucleoid conformations corresponding to different physiological and developmental stages (Eltsov and Zuber, 2006). In an attempt to visualize *Epulopiscium* cellular components, most especially the unusual conformation of the nucleoids and their association with cell membrane invaginations, I attempted cryofixation, freeze substitution, and transmission electron microscopy of *Epulopisicum* sp. Type B. As *Epulopiscium* is not yet in culture, I fixed and used samples obtained from the host fish *Naso tonganus.*

Materials and Methods

Conventional Fixation

Naso tonganus was collected by spearfishing from outer reefs around Lizard Island, Great Barrier Reef, Australia in March 2005. The intestinal tract of one fish that was shot at 11 AM was excised and 1 ml of the gastrointestinal contents was fixed using a variety of conventional chemical fixation protocols. All chemicals not otherwise noted were obtained from Sigma Aldrich, Saint Louis, MO. For investigating morphology, I fixed *Epulopiscium* cells in 9 ml of sodium cacodylate fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1M NaCac, all fixatives EM 2 Grade, Electron Microscopy Sciences, Hatfield, PA) for 30 min at room temperature. For use for immunogold staining, I fixed *Epulopiscium* cells in 9 ml of sodium phosphate fixative (4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer) for 1 hr at 4° C. After fixation, the samples were washed three times in the appropriate buffer and dehydrated in ethanol (Immunogold: 20% EtOH, 4° C, 30 min, 50% EtOH, -20° C, 30 min, 70% EtOH, -20° C; Morphology: 20% EtOH, 4° C, 10 min, 50% EtOH, 4° C, 10 min, 70% EtOH, -20° C). The samples were stored in 70%

EtOH at the appropriate temperature until arriving at Australian National University 2 days later.

Cryofixation

Two *Naso tonganus* were caught by spearfishing at 8 AM. These fish had no puncture wounds in the abdominal cavity. They were then placed inside an ice chest without coolant (Esky, Australia) and flown to Australian National University (ANU) in Canberra. Total time between spearing and dissection of the host fish was ~8 hours (flight time as well as additional travel and set-up time combined). Previous reports indicated that *Epulopiscium* are still viable inside the dead host for this length of time (Clements & Angert, personal communication). Upon arriving at ANU in Canberra, an anaerobic bag (I2R Model X-27-27, Instruments for Research and Industry, Cheltenham, PA) was flushed with CO_2 twice and a GasPack (Hydrogen + CO_2, BBL Gas Pack System, Becton Dickinson Co., Cockeysville, MD) was sealed inside the bag along with dissection tools, sample vials and the two host fish. The fish were dissected inside the bag and the gastrointestinal contents were placed inside stoppered glass vials (Wheaton Scientific, Millville, NJ). A portion of the sample (0.5 ml from a total of 3 ml per fish) was used for Trypan Blue staining (1:1 with 0.8 mM in PBS) to test the integrity of the cells.

After verifying that some cells were still intact, I used the propane jet method of cryofixation with the cryoprotectant sucrose (anaerobic, final concentration 0.2M) (Bozzola and Russell, 1999; Ding *et al.*, 1991). Briefly, 100 μl of cell suspension in cryoprotectant was extracted from the stoppered vial via a 17-gauge needle (diameter of ~1000 microns) and syringe (Sigma-Aldrich, Saint Louis, MO). The cell suspension was placed between the top hat copper plates without a spacer and the top hats were clamped into the jet freezer (Bal-Tec JFD 030 Twin jet freezer, Canonsburg, PA). The sample was vitrified by exposure to a blast of liquid propane that had been

chilled with liquid nitrogen. The frozen sample was then released from the top hats into a liquid nitrogen bath. Freeze substitution of samples was carried out in an automated freeze substitution apparatus (Leica EM AFS2 + EM FSP, Bannockburn, IL) with the following program: 24 hours at -90° C, 12 hours in new solution warming to -20° C, and 4 hours in new solution warming to 4° C. Freeze substitution was done in acetone (immunogold samples) or acetone with 2% uranyl acetate/2% Osmium tetraoxide (morphology samples). The samples intended for immunogold labeling were then placed in 1:1 acetone:EtOH for 10 min, and then washed twice in 100% EtOH for 10 min.

Embedding

Samples fixed by both conventional and cryo methods for immunogold were embedded at -20° C as follows: 3:1 EtOH: LR White embedding resin (Electron Microscopy Sciences, Hatfield, PA) for 30 min, 2:1 for 1 hr, 1:1 for 1 hr, 1:2 for 1 hr, 1:3 for 1 hr and pure LR White overnight. Samples were then transferred to fresh resin with accelerator, positioned in gelatin capsules (Electron Microscopy Sciences, Hatfield, PA) and cold polymerized via UV overnight (2X15W UV bulbs with the samples positioned 15cm below the light source and the chamber held near -20ºC with dry ice).

Samples fixed by both conventional and cryo methods for examination of morphology were embedded as follows: 8:1 Acetone: Epon Araldite (Electron Microscopy Sciences, Hatfield, PA) 30 min, RT, 4:1 for 30 min, 2:1 for 1 hr, 1:1 for 12 hrs, 1:2 for 3 hrs, 1:3 for 4 hrs, 1:4 for 5 hrs, 100% Epon Araldite for 12 hrs. Accelerator was then added to the Epon Araldite and this mixture was infiltrated for 5 hours after which samples were positioned into a flat embedding mold and cured at 70ºC overnight.

CryoSEM

A portion of the conventionally fixed glutaraldehyde sample was used for cryo-SEM in collaboration with Dr. Ray Roberts at ANU. After rehydration overnight in PBS, preparation for viewing was done at ANU as previously reported (Roberts and Evans, 2005).

Sample Examination

Both thick (~2 μm) and thin (~0.4 μm) sections from all four types of blocks were taken with an ultramicrotome (LKB Ultrotome V, Sweden). Thick sections were stained with Crystal Violet (Difco, Becton Dickinson, Sparks, MD, USA) and viewed on an Olympus BX61 epifluorescence microscope, with 40x UPlanFl (N.A. 0.75) and 100x UPlanApo (N.A. 1.35) objectives. Images were acquired using a Cooke SensiCam (Cooke Corp., Romulus, MI) and Slidebook software (Intelligent Imaging Inc., Denver, CO). Thin sections were placed on grids (Electron Microscopy Sciences, Hatfield, PA) stained with 2% uranyl acetate for 10 min, washed twice, stained with 3% lead citrate for 5 min, washed again and dried on filter paper. The electron microscope used for micrographs was a Phillips EM-201 equipped with a Gotan model 780 camera. Photos were taken at 100 KV.

Results

The anaerobic bag set up used for dissection is shown in Figure 1A. Thick sections were examined in order to find *Epulopiscium* cells within the Epon Araldite embedded sample. Several protists of a similar size as *Epulopiscium* were found and were apparently well infiltrated (Figure 1B). Few *Epulopiscium* cells were found and those that were examined had poor infiltration such that the section disintegrated prior to viewing. Several blocks were sent to the University of Guelph for examination by Dr. Terry Beveridge. However all investigations into the Epon Araldite blocks, regardless of the fixation type, indicated that the resin had not completely infiltrated the cells.

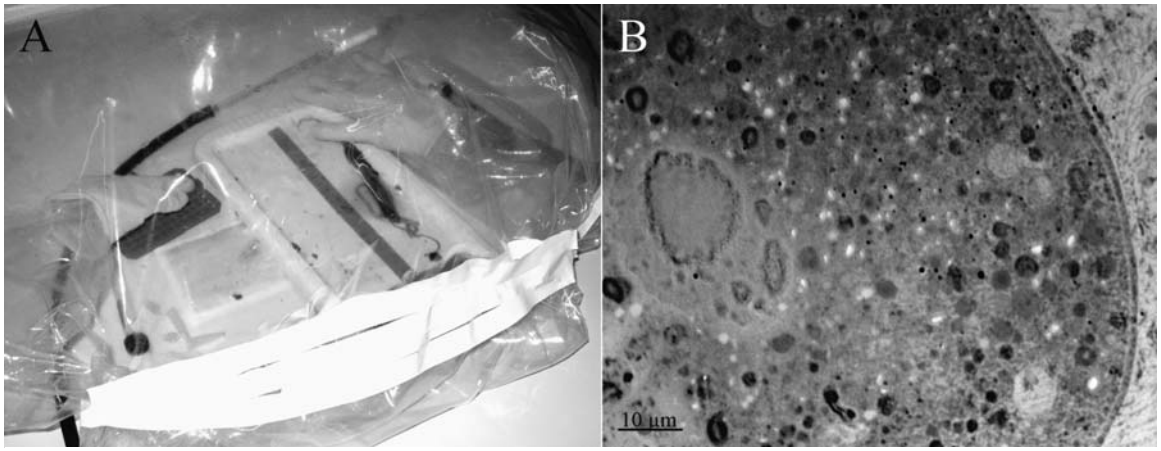

Figure 1. All dissections were performed inside an anaerobic bag in order to maintain the viability of *Epulopsicium* cells (A). Thick sections, from a cryofixed sample which was embedded for morphology, reveal what appear to be large protists (B).

Due to lack of contrast and the difficulty of finding the appropriate cells within the sample, the LR White samples were not examined.

Cryo-SEM images depicted the protist most likely viewed in the thick sections, *Balantidium jocularum* (Figure 2A). This organism preys on the large *Epulopiscium* cells (Grim, 2006). Also notable from the Cryo-SEM was the consistent longitudinal indentation on the *Epulopiscium* cells (Figure 2B). The cells appeared coated with the viscous gastrointestinal contents, including other bacteria (Figure 2C, 2D).

Discussion

The failure to obtain useful data from the samples prepared for TEM was unfortunate but not unexpected. To my knowledge, this is the first attempt to use cryofixation followed by TEM to examine the ultrastructure of an uncultured organism. The travel time between the field site and the cryofixation facilities alone placed a great deal of risk in the trial as it could not be repeated nor the blocks examined prior to leaving ANU to come back to Cornell. However, given the results indicating poor infiltration, several steps could be taken to minimize the likelihood of failure if this experiment

were undertaken again. First, the freeze substitution should be extended longer. No bacterium of a comparable size has been successfully processed in this way and therefore a more conservative estimate of exchange times between vitrified cells and the dehydrating solvent is in order. Also, newly ordered resin should be mixed on site

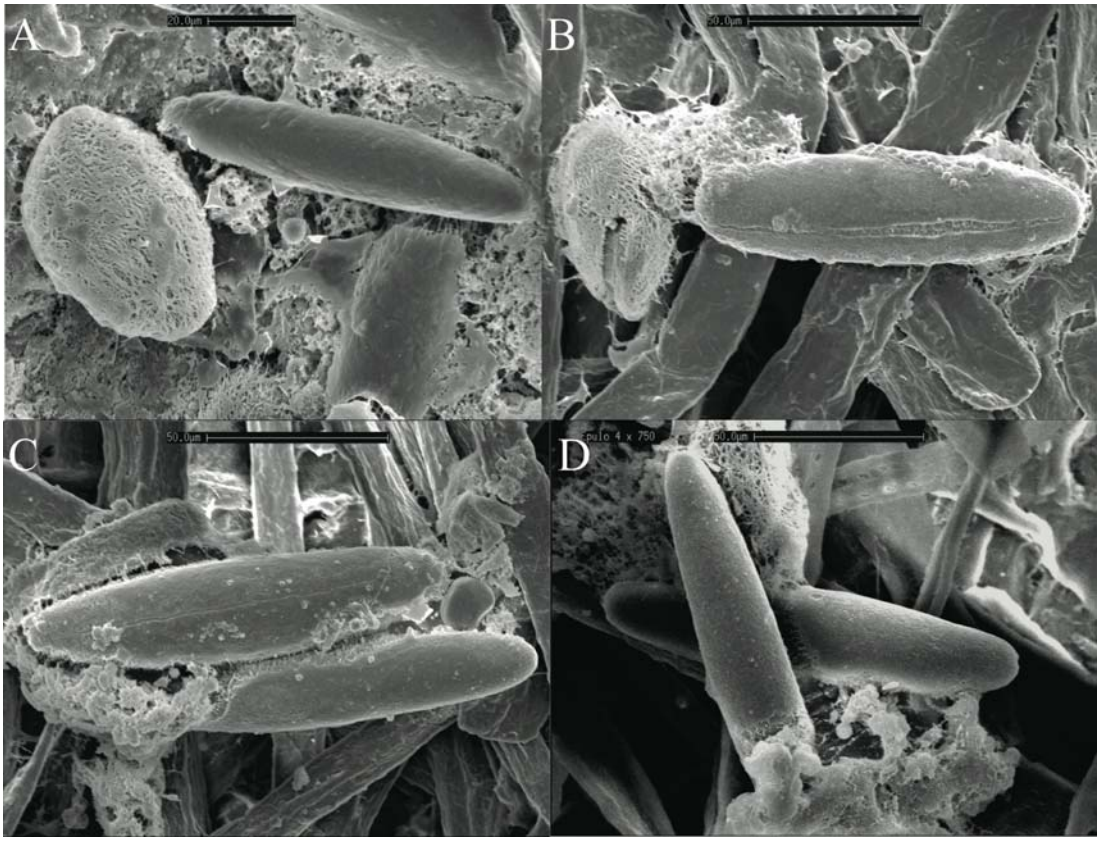

Figure 2. Cryo-SEM images of the contents of the intestinal lumen of *N. tonganus*.

prior to infiltration and these times should also be extended. Because of the particular issues with these samples, we cannot evaluate the quality of fixation for the object of interest, but another possibility for future experiments is using high pressure cryofixation which has depths of vitrification much greater than propane jet (M.V. Parthasarathy, personal communication).

The Cryo-SEM work raised several questions. The protist cilia are intact but the *Epulopiscium* cells appear to have no flagella, nor even broken ends of flagella along

the cell surface. These cells come from a fish speared at 11 AM, a time when generally *Epulopiscium* daughter cells have emerged from the mother cell and should be vigorously motile (E.R. Angert, personal communication). One possibility is that, shortly after emerging, flagella are assembled on the surface of the daughter cell and these cells were fixed just prior to that developmental stage. Another intriguing observation from the SEM images is the indentation along the length of the *Epulopiscium* cells. This may certainly be an artifact arising from dessication and deformation of the cell wall as these cells spent almost 2 full days in ethanol prior to viewing. Also the process of Cryo-SEM is designed for hydrated samples, quickly frozen, which retain their native shape without distortion, whereas these had been dehydrated, rehydrated, and the vitrified. However, the appearance of a consistent indentation in many of the cells viewed indicates that this may represent some sort of pressure point when the cell encounters certain mechanical stressors. This might be an advantageous system for allowing rapid and efficient lysis when mother cells release live offspring. Although none of the results from this work are conclusive, it is clear that a better understanding of the subcellular organization of these unusual bacteria is urgently needed.

Acknowledgements

Dr. Ray Roberts performed the Cryo-SEM at Australian National University.

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APPENDIX C

METHANOREGULA BOONEI GEN. NOV., AN ACIDIPHILIC METHANOGEN ISOLATED FROM AN ACIDIC PEAT BOG

Suzanna Bräuer, Rebekah J. Ward, Joseph Yavitt, and Stephen Zinder.

Abstract

A novel acidiphilic hydrogenotrophic methanogen, designated strain 6A8 was isolated from McLean Bog, an ombrotrophic, or rain-fed, kettle hole bog of pH 4.0-4.5 located near Ithaca, NY, USA. Cells were dimorphic, consisting of a thin rod (0.2-0.3 μm diameter and 0.8-3.0 μm long) and irregular coccoid cells 0.3-0.8 μm in diameter. The culture utilized H_2/CO_2 to produce methane, but did not utilize formate, acetate, methanol, ethanol, isopropanol, or trimethylamine as methanogenic substrates. To our knowledge, this strain is the most acidiphilic and salt sensitive methanogen in pure culture. Optimal growth conditions were around pH 5.1 and from 0.002 to less than 0.29% (0.4 to < 50 mM) NaCl. Acetate, coenzyme M and vitamins and yeast extract were required for growth. The closest relative in pure culture, *Methanospirillum hungatei* JF1, shares only 90% sequence identity with the 16S rRNA gene sequence of strain $6A8^T$. In addition, many other genotypic and phenotypic characteristics distinguish this new methanogen from *Methanospirillum hungatei,* including cell morphology, pH optimum, optimal salt concentrations, G+C content of the DNA, lack of cell sheath, lack of motility, and inability to use formate or alcohols. Based on these differences, it is proposed that a new genus and species be established for this organism, *Methanoregula boonei*. The type strain is 6A8.

Materials and Methods

Electron microscopy. Cells were concentrated by centrifugation, washed and resuspended in deionized water. A drop (approx. 5 μL) of the concentrated cells was mixed with a drop of bacitracin (100 μg/mL) and allowed to settle onto Formvar-

coated copper grids (100 mesh) for 5 minutes before staining with 2% uranyl acetate (pH 6.5) for a few seconds. The electron microscope used for low-resolution micrographs was a Phillips EM-201 equipped with a Gotan model 780 camera and that used for high-resolution micrographs was a Phillips Technai 12 Biotwin, equipped with a Gatan Multiscan model 791 camera and Digital Micrograph software. Photos were taken at 100 KV.

Results

Cell morphology and architecture. Cells of 6A8^T were dimorphic, consisting of a thin rod (0.2-0.3 μm diameter and 0.8-3.0 μm long) and irregular coccoid cells 0.3-0.8 μm in diameter. Asymmetric cell division was sometimes observed in rod-shaped cells, but cell division was not observed in coccoid cells (Bräuer *et al.*, 2006b). Autofluorescence characteristic of significant quantities of F_{420} in cells was not observed. Transmission electron micrographs of negatively-stained cell cultures of strain 6A8^T (Figure 1) showed that both morphotypes had similar cell wall structures, resembling the S-layer cell walls found in most *Archaea*. Both morphotypes had similar "notches," which are attributed to fracturing of the S-layer by the flattening of the cells during preparation, pilus-like filamentous appendages of similar diameter (9-11 nm), and some preparations revealed the presence of dark spots, which might be attributed to storage granules. The diameters of the filaments are at the lower boundary of those for archaeal flagella (10-14 nm) (Thomas *et al.*, 2001), but motility by these cells has not been observed.

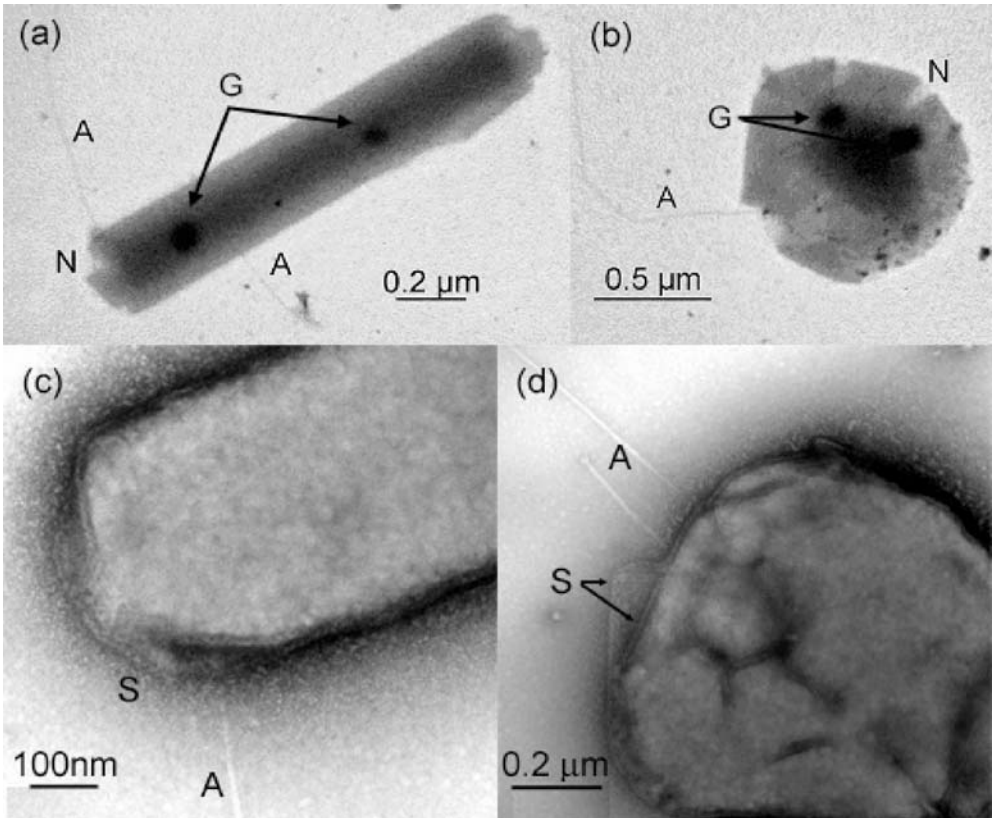

Figure 1. Electron micrographs of a uranyl acetate-stained rod-shaped cells (a, c) or coccoid cells (b,d) from strain 6A8^{T} cultures. a, b, Low-resolution micrographs showing notches (N) in the cell wall, putative storage granules (G), and pilus-like appendages (A), and c, d, higher resolution micrographs showing subunit structure (S) at the periphery of the outer layer and pilus-like appendages (A).

APPENDIX D

ANTIMICROBIAL BEHAVIOR OF SEMIFLUORINATED-QUATERNIZED TRIBLOCK COPOLYMERS AGAINST AIRBORNE AND MARINE MICROORGANISMS

Daewon Park, Rebekah J. Ward, Craig J. Weinman, Sitaraman Krishnan, Marvin Paik, Karen E. Sohn, John A. Finlay, Maureen E. Callow, James A. Callow, Dale L. Handlin, Carl L. Willis, Daniel A. Fischer, Esther R. Angert, Edward J. Kramer, and Christopher K. Ober

Abstract

Semifluorinated-quaternized triblock copolymers (SQTCs) were synthesized by chemical modification of polystyrene-*block*-poly(ethylene-*ran*-butylene)-*block*-polyisoprene ABC triblock copolymers. Surface characterization of the polymers was performed by X-ray photoelectron spectroscopy (XPS) and near-edge X-ray absorption fine structure (NEXAFS) analysis. *Ulva* seaweed spores strongly adhered to the surface of the SQTCs suggesting the presence of N^+ atoms on the surfaces. Additionally, the surfaces of the SQTCs showed bacteriostatic activity against the bacterium *Staphylococcus aureus* with 100 % inhibition of growth and indeterminate bactericidal activity against the marine bacterium *Cobetia marina*.

Materials and Methods

Antibacterial Tests: Cell Growth

Staphylococcus aureus of 100 μL was incubated in Trypticase Soy Broth (TSB, 5 mL; per liter: 3 g of soy meal peptone, 17 g of casein peptone, 2.5 g of glucose, 5 g of NaCl, and 2.5 g of dipotassium hydrogen phosphate) at 37 ^{o}C for 4 h. The cells were centrifuged at 5000 rpm for 1 min using a microcentrifuge (Eppendorf 5415C), and the precipitate was resuspended in 1 mL of sterile filtered water.

Cobetia marina (ATTC 25374) was grown overnight in broth from a single isolated colony from a plate. Difco marina broth and Difco marine agar were used for growth. The cells were innoculated into fresh marine broth (100 uL into 4.9 ml) and grown for 5 hours. Then, 1 mL of exponential culture was placed in an Eppendorf tube, centrifuged at maximum speed for a clinical centrifuge and resuspended in 1 mL of sterile water.

Antibacterial Tests: Colony Counts

Aqueous suspensions of *S. aureus* with concentrations of ~10^6 cells/mL were sprayed on the test surfaces, dried in air for 2 min and placed in sterile Petri dishes. They were covered by molten agar-containing TSB (1.5 % w/v of agar), allowed to solidify and then incubated at 37º C overnight. The number of bacterial colonies was counted using a colony counter.

Antibacterial Tests: Live/Dead BacLight Bacterial Viability Assay

Live/Dead Bacterial Viability Kit was obtained from Molecular Probes, Inc. Equal volumes of SYTO 9 and propidium iodide (PI) were mixed thoroughly in a microcentrifuge tube. The BacLight dye mixture (30 μL) was added to 1 mL of the cell suspension, which was then sprayed on the test surfaces. Immediately after the spraying, the test surfaces were covered with glass coverslips, sealed with finger nail polish to avoid dessication, and incubated in the dark for 15 min. Phase-contrast and fluorescence microscopy were performed, within 1hr after spraying, using an Olympus BX61 epifluorescence microscope with a 100× UPlanApo (N.A.135) objective. The microscope was equipped with filter cubes for viewing SYTO 9 and PI fluorescence. Glass microscope slides were used as controls.

Results and Discussion

Antibacterial Behavior of SQTC-F8H6Br-H6Br against S. aureus and C. marina bacteria

The antibacterial activity of SQTC-F8H6Br-H6Br was determined by comparing the number of bacterial colonies of *S. aureus* and *C. marina* grown on coated test surfaces relative to plain glass slides. As shown in Figure 1, the mean value of bacterial colonies of *S. aureus* grown on plain glass slides after overnight incubation was ca. 125 colonies/cm^2. In contrast, less than 4 bacterial colonies, if any, grew on surfaces coated with SQTC-F8H6Br-H6Br, which reflected high antibacterial activity. We were unable to perform colony count assays on *C. marina* due to the lack of adherence of the bacterial cells to the surfaces which lead to inconsistent growth. Further study using Live/Dead *Bac*Light Bacterial Viability Assay provided more information regarding the antibacterial behavior of SQTC-F8H6Br-H6Br surface on both *S. aureus* and *C. marina*.

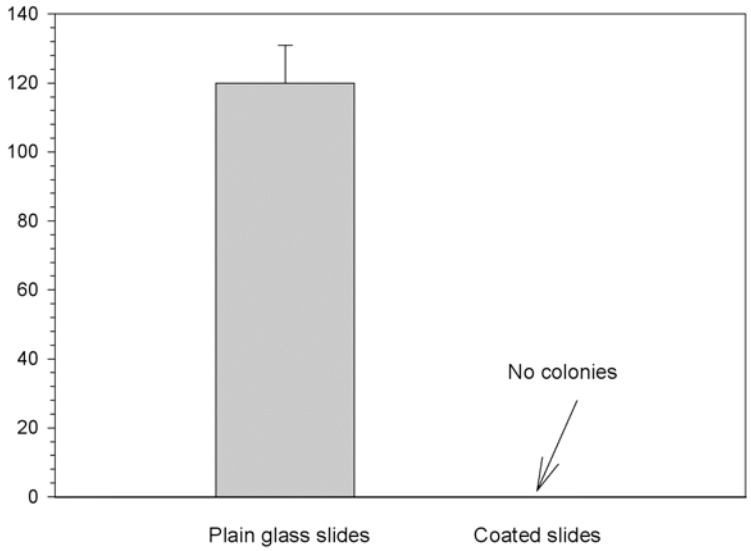

Figure 1. Antibacterial activities of SQTC-F8H6Br-H6Br against *S. aureus*.

Figures 2 shows the fluorescence microscopy images of the Live/Dead Assay at 45 min. Figure 3 depicts the percentage of damaged cells determined by comparison of the live/intact cells (green) and dead/damaged cells (red). At first, the images on *S. aureus* showed no discernable difference between control and coated surfaces (Figures 6A and 6B). However, analysis of the images for total fluorescence by software (Slidebook software, Intelligent Imaging Inc.) showed more cells (ca. 2 fold) on the coated surfaces were damaged with no significant changes in an hour (Figure 3 Left). This result is quite different from our previous study by Krishnan et al (2006). In this study, surfaces of polystyrene$_{62K}$-*block*-poly-(4-vinyl pyridine)$_{66K}$, (PS-*b*-P4VP) block copolymer fluorinated with F8H6Br-H6Br showed almost 100 % bactericidal activity against *S. aureus.* This is in stark contrast to the bacteriostatic nature of our SQTC-F8H6Br-H6Br surfaces. One possible explanation of the observed differences in antibacterial activity is the relative amount of quaternized nitrogen in each of the two polymers. In 1 g of 59.6 % aminated PS-*b*-P(E/B)-*b*-PI there is 4.0 mmol nitrogen. Based on N 1s XPS spectra, 70 % of this nitrogen was quaternized which corresponds to 2.8 mmol quaternized nitrogen per 1 g of polymer. Meanwhile, the total amount of quaternized nitrogen in 1 g of PS-*b*-P4VP is 4.66 mmol. Thus, more quaternized nitrogen on the surfaces seems to act as a favorable factor for enhanced bactericidal activity. The images of *C. marina* showed that cells on the SQTC-F8H6Br-H6Br coated surface were damaged, however the control also showed significant damage (Figures 2C and 2D). For *S. aureus*, the percentage of damaged cells seemed to increase constantly while the damaged cells on the controls had no major changes but rather small fluctuations (Figure 7, left). The differences in damage to *C. marina* between the control and the polymer-coated surfaces were negligible (Figure 3, right).

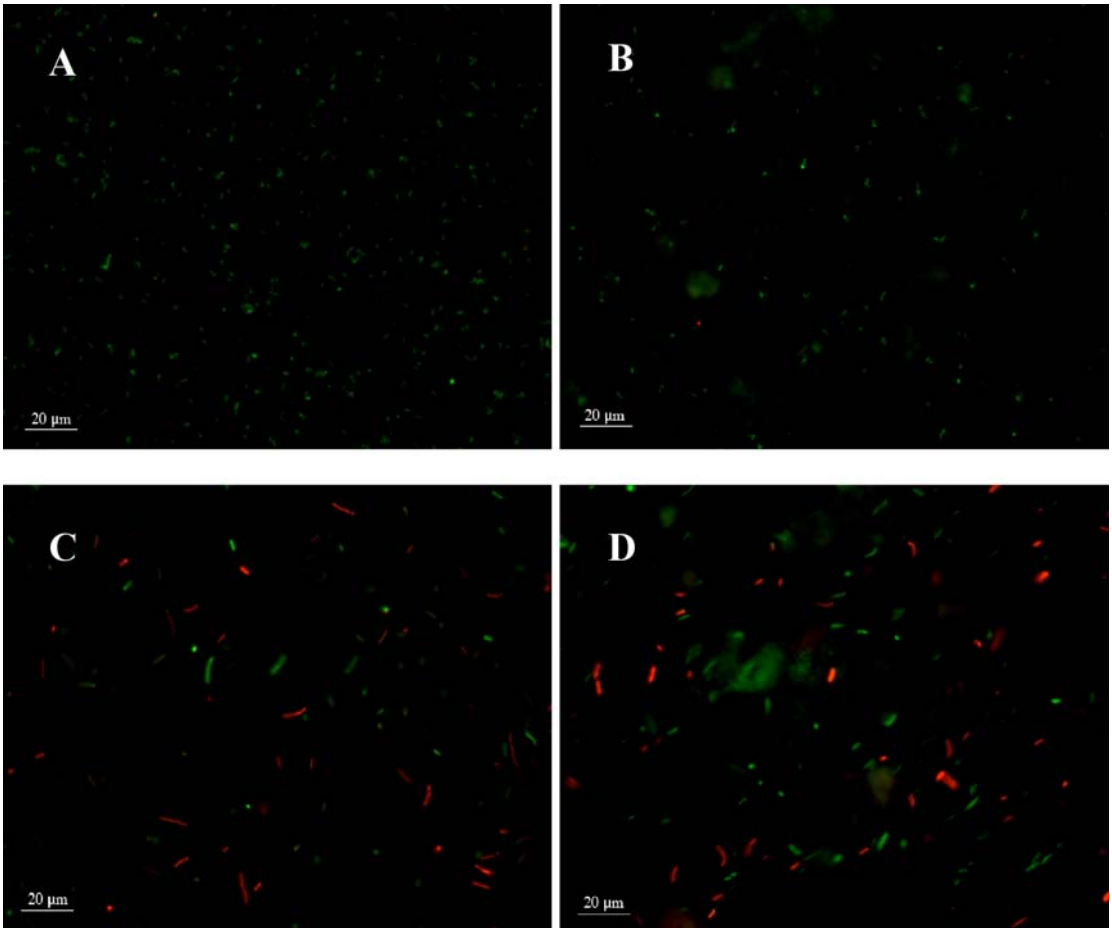

Figure 2. Images of *S. aureus* on (A) glass control; (B) SQTC-F8H6Br-H6Br coated surfaces and images of *C. marina* on (C) glass control; (D) SQTC-F8H6Br-H6Br coated surfaces by Live/Dead *Bac*Light Bacterial Viability Assay. Cells with intact cell membranes are stained green, and those with damaged membranes are stained red.

Thus, the antibacterial activity of SQTC-F8H6Br-H6Br surfaces seems to impair growth for *S. aureus* and had indeterminate effects on *C. marina*.

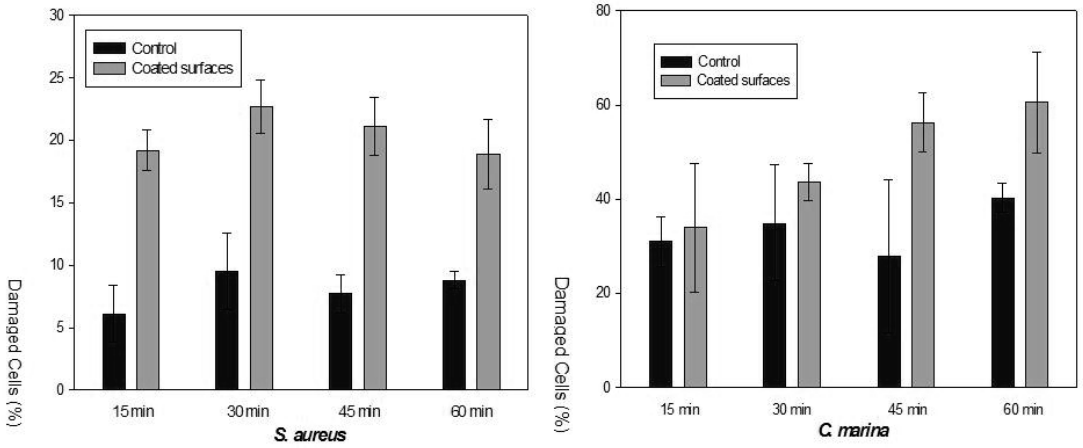

Figure 3. Antibacterial behavior of SQTC-F8H6Br-H6Br against *S. aureus* and *C. marina* by Live/Dead *Bac*Light Bacterial Viability Assay.

APPENDIX E

SURFACES OF FLUORINATED PYRIDINIUM BLOCK-COPOLYMERS WITH ENHANCED ANTIBACTERIAL ACTIVITY

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Abstract: Polystyrene-*block*-poly(4-vinylpyridine) copolymers were quaternized with 1-bromohexane and ω-6-perfluorooctyl-bromohexane. Surfaces prepared from these polymers were characterized by contact angle measurements and near-edge X-ray absorption fine structure spectroscopy. The fluorinated pyridinium surfaces showed enhanced antibacterial activity compared to their non-fluorinated counterparts. Even a polymer with a relatively low molecular weight of the pyridinium block was highly active. Antibacterial activity was found to be related to the molecular composition and organization in the top 2–3 nm of the surface, and increased with an increase in the hydrophilicity and pyridinium concentration of the surface.

Materials and Methods

Antibacterial tests

Aqueous suspensions of *Staphylococcus aureus* with concentrations of ~10^6 cells/mL were sprayed using a chromatography sprayer on the test surfaces. The sprayed surfaces were dried in air for ca. 2 min. Agar gel containing caesin peptone (17 g/L), soy meal peptone (3 g/L), glucose (2.5 g/L), NaCl (5 g/L) and dipotassiumhydrogenphosphate (2.5 g/L) as nutrients, was poured on the slides, allowed to solidify, and incubated at 37 °C overnight. The number of bacterial colonies was counted using a colony counter. Corning glass slides that were not coated with the quaternized polymers were uses as controls. Typical density of colonies on the glass controls were 100–150 per cm^2 of the surface.

Results and Discussion

Antibacterial Assay

As seen in Figure 1, a large number of bacterial colonies formed on the glass slide, which is not expected to have any bactericidal activity. Assuming that the same

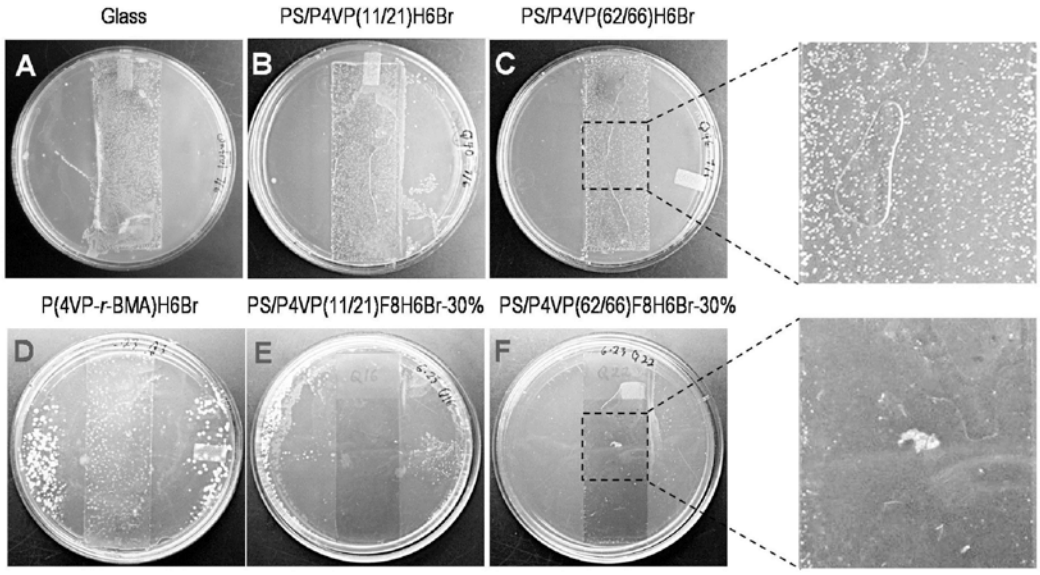

Figure 1. Photographs of test surfaces after 12 h of incubation with *S. aureus* cells under agar gel in a bacterial growth medium at 37 °C. The width of the glass microscope slides in the figure is 1 inch.

number of *S. aureus* cells were sprayed onto the glass control and test surfaces, and that the cells were killed on contact with the surface (within a few minutes after spraying the bacterial suspension), the relative number of colonies on the glass and test surfaces represents the fraction of the sprayed cells that remained viable on the test surfaces. It was observed that while the surfaces of polymers quaternized with 1-bromohexane (B and C) had a large number of bacterial colonies, only about 20 % lower that that on the control glass surface, the fluorinated pyridinium polymers (E

and F) showed almost 100 % reduction in the number of colonies (cf. Figure 2). Thus, although the number of monomer units in the pyridinium blocks were the same, quaternization with the semifluorinated alkyl bromide resulted in a large increase in the bactericidal activity. The enhanced antibacterial activity can be attributed to the differences in the chemical composition and molecular organization within the top few nanometers of the surfaces, and possibly due to a greater ability of the rigid and highly hydrophobic perfluoroalkyl helices to disrupt the bacterial cell membrane. The former was investigated using NEXAFS spectroscopy and contact angle measurements. The colony count for the high molecular weight poly(N-hexyl pyridinium bromide-*ran*-*n*-butyl methacrylate) (D) was about 60 % lower than that on glass. Thus, although the molecular weight of this polymer was significantly higher (300 kDa), its bactericidal efficiency was lower than that of the PS/P4VP polymer, with 21 kDa P4VP block, quaternized using w-6-perfluorooctyl-bromohexane. This suggests that in addition to molecular weight, the molecular organization at the surface played a crucial role in antibacterial activity.

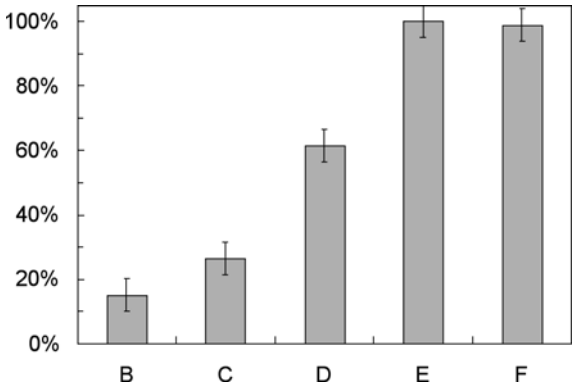

Figure 2. Percentage reduction in the number of *S. aureus* colonies relative to the colony count on glass slide. The colonies were counted after 12 h of incubation in the growth medium. (B) PS/P4VP(11/21)H6Br, (C) PS/P4VP(62/66)H6Br, (D) P(4VP-*r*-BMA)H6Br, (E) PS/P4VP(11/21)F8H6Br-30%, and (F) PS/P4VP(62/66)F8H6Br-30%.